PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/16, 38/18, C07H 21/04, C07K 1/12, 14/00, 14/435, 14/475, C12N 15/00, 15/09, 15/12 — A1

(11) International Publication Number: WO 95/26746

(43) International Publication Date: 12 October 1995 (12.10.95)

(21) International Application Number: PCT/US95/03776

(22) International Filing Date: 30 March 1995 (30.03.95)

(30) Priority Data:

08/221,768	31 March 1994 (31.03.94)	US
08/252,628	31 May 1994 (31.05.94)	US
08/321,488	12 October 1994 (12.10.94)	US
08/347,780	30 November 1994 (30.11.94)	US

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: COMPOSITIONS AND METHODS FOR STIMULATING MEGAKARYOCYTE GROWTH AND DIFFERENTIATION

(57) Abstract

Disclosed are novel proteins, referred to as megakaryocyte growth and development factors (MGDFs; also generally referred to as Mpl ligands), that have a biological activity of stimulating the growth of megakaryocytes and augmenting the differentiation or maturation of megakaryocytes, ultimately to result in the production of platelets. MGDF derivatives comprising MGDF molecules attached to water-soluble polymers, such as polyethylene glycol, are also disclosed, along with methods for their preparation. Also disclosed are processes for obtaining the MGDFs in homogeneous form from natural sources and producing them by recombinant genetic engineering techniques from mammals, including humans.

COMPOSITIONS AND METHODS FOR STIMULATING MEGAKARYOCYTE GROWTH AND DIFFERENTIATION

Field of the Invention

The present invention relates to novel proteins, referred to herein synonymously as Mpl ligands or MGDFs, that stimulate the growth of megakaryocytes and augment the differentiation or maturation of megakaryocytes, with the ultimate effect of increasing the numbers of platelets. Also provided are processes for obtaining the proteins in homogeneous form from natural sources and producing them by recombinant genetic engineering techniques.

In another aspect, the present invention broadly relates to a novel class of MGDF derivatives wherein an MGDF molecule is attached to a water soluble polymer, and methods for preparing such molecules. In yet another aspect, the present invention relates to MGDF derivatives wherein an MGDF molecule is attached to one or more polyethylene glycol ("PEG") groups, and methods of their preparation.

Background of the Invention

At least two broad areas of research are involved in the present invention. The first relates to the development of megakaryocytes and subsequent production of platelets, and the second relates to a polypeptide member of a growth factor receptor family, referred to herein as the Mpl receptor, and ligands thereof. Each of these areas of research will now be outlined in the following.

A. Platelet Production from Megakaryocytes

Blood platelets are circulating cells that are crucial for the prevention of bleeding and for blood coagulation. Megakaryocytes are the cellular source of platelets and arise from a common bone marrow precursor cell which gives rise to all hematopoietic cell lineages. This common precursor cell is known as the pluripotent stem cell or PPSC.

A hierarchy of megakaryocytic progenitor cells has been defined based on the time of appearance and size of megakaryocyte (MK) colonies appearing in *in vitro* culture systems in response to appropriate growth factors. The burst-forming unit megakaryocyte (BFU-MK) is the most primitive megakaryocyte progenitor cell. BFU-MK are thought ultimately to produce numerous colony forming unit megakaryocytes (CFU-MK), which are more differentiated MK progenitor cells.

As the MK cells undergo subsequent differentiation, they lose the ability to undergo mitosis but acquire an ability to endoreduplicate. Endoreduplication (or endomitosis) is the phenomenon in cells of nuclear division in the absence of cell division. Endoreduplication ultimately results in an MK which is polyploid. Further MK maturation results in acquisition of cytoplasmic organelles and membrane constituents that characterize platelets.

Platelets are produced from mature MK's by a poorly defined process that has been suggested to be a consequence of MK physical fragmentation, or other mechanisms. Observations of extensive membranous structures within megakaryocytes has led to a model of platelet formation in which a demarcation membrane system outlines nascent platelets within the cell body. Another model of platelet formation has developed from observations that megakaryocytes will form long

cytoplasmic processes constricted at platelet-sized intervals from which platelets presumably break off due to blood flow pressures in the marrow and/or in the lung. These cytoplasmic processes were termed proplatelets by Becker and DeBruyn to reflect their presumed precursor role in platelet formation. *See* Becker and DeBruyn, *Amer. J. Anat.* **145**: 183 (1976).

FIG. 1 presents an overview of the various precursor cells involved in megakaryocyte and platelet development. The cell at the far left-hand side of the figure represents a PPSC, and the additional cells to the right of the PPSC in the figure represent BFU-MK, followed by CFU-MK. The cell that is undergoing endoreduplication, which is located immediately to the right of the PPSC in the figure, is a mature megakaryocyte cell. As a result of endomitosis, this cell has become polyploid. The next structure to the right includes long cytoplasmic processes emerging from the polyploid nucleus of the mature megakaryocyte cell. In the far right-hand side of the figure are shown a number of platelets that have been produced by fragmentation of the cytoplasmic processes.

The following is a summary of some prior publications relating to the above description of megakaryocyte maturation and the production of platelets:

1. Williams, N. and Levine, R.F., *British Journal of Haematology* **52**: 173-180 (1982).

2. Levin, J., *Molecular Biology and Differentiation of Megakaryocytes*, pub. Wiley-Liss, Inc.: 1-10 (1990).

3. Gewirtz, A.M., *The Biology of Hematopoiesis*, pub. Wiley-Liss, Inc.: 123-132 (1990).

4. Han, Z.C., et al., *Int. J. Hematol.* **54**: 3-14 (1991).

5. Nieuwenhuis, H.K. and Sixma, J., *New Eng. J. of Med.* **327**: 1812-1813 (1992).

6. Long, M., *Stem Cells* **11**: 33-40 (1993).

B. <u>Regulation of Platelet Formation</u>

A large body of data generated in many laboratories indicates that platelet production is regulated by humoral factors. The complexity of this biological process was not originally appreciated and currently it appears that a number of human growth factors possess this capability.

Megakaryocyte regulation occurs at multiple cellular levels. A number of cytokines enhance platelet production by expanding the progenitor cell pool. A second group of humoral growth factors serves as maturation factors acting on more differentiated cells to promote endoreduplication. In addition, there appear to be two independent biofeedback loops regulating these processes.

Several lineage nonspecific hematopoietic growth factors exert important effects on MK maturation. Granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3), IL-6, IL-11, leukemia inhibitory factor (LIF), and erythropoietin (EPO) each individually promote human MK maturation *in vitro* as determined by their effects on MK size, number, or ploidy. The MK maturational effects of LIF, IL-6, and IL-11 are either partially (LIF and IL-6) or totally (IL-11) additive to those of IL-3. Such data from these prior publications suggested that combinations of cytokines may be necessary to promote MK maturation *in*

vivo.

The following is a summary of some prior publications relating to the regulation of megakaryocyte and platelet production:

7. Hoffman, R. et al., *Blood Cells* **13**: 75-86 (1987).

8. Murphy, M.J., *Hematology/Oncology Clinics of North America* **3** (3): 465-478 (1988).

9. Hoffman, R., *Blood* **74** (4): 1196-1212 (1989).

10. Mazur, E.M. and Cohen, J.L., *Clin. Pharmacol. Ther.*, **46** (3): 250-256 (1989).

11. Gewirtz, A.M. and Calabretta, B., *Int. J. Cell Cloning* **8**: 267-276 (1990).

12. Williams, N., *Progress in Growth Factor Research* **2**: 81-95 (1990).

13. Gordon, M.S. and Hoffman, R., *Blood* **80** (2): 302-307 (1992).

14. Hunt, P. et al., *Exp. Hematol.* **21**: 372-281 (1993).

15. Hunt, P. et al., *Exp. Hematol.* **21**: 1295-1304 (1993).

It has also been reported (see reference 16) that human aplastic serum contains a megakaryocyte colony stimulating activity distinct from IL-3, granulocyte colony stimulating factor, and factors present in lymphocyte-conditioned medium. However, the molecule responsible for this activity was neither isolated nor characterized in the prior art.

16. Mazur, E.M., et al., *Blood* **76**: 290-297 (1990).

C. <u>The Mpl Receptor</u>

The myeloproliferative leukemia virus (MPLV) is a murine replication-defective retrovirus that causes acute leukemia in infected mammals. It has been discovered that a gene expressed by MPLV consists of a part of the gene that encodes the retroviral envelope (or external protein coat) of the virus fused to a sequence that is related to the cytokine receptor family, including the receptors for GM-CSF, G-CSF, and EPO.

Expression of the MPLV gene described above has the interesting biological property of causing murine progenitor cells of various types to immediately acquire growth factor independence for both proliferation and terminal maturation. Moreover, some cultures of bone marrow cells acutely transformed by MPLV contained megakaryocytes, suggesting a connection between the MPLV gene and megakaryocyte growth and differentiation.

It is now recognized that the MPLV viral gene (referred to as v-Mpl) has a homolog in mammalian cells, which is referred to as a cellular Mpl gene (or c-Mpl). Using v-Mpl-derived probes, a cDNA corresponding to the human c-Mpl gene was cloned. *See* PCT published application WO 92/07074 (published April 30, 1992; discussed below). Sequence analysis has shown that the protein encoded by the c-Mpl gene product belongs to the highly conserved cytokine receptor superfamily, just like the homologous v-Mpl gene product.

This cellular gene, c-Mpl, is thought to play a functional role in hematopoiesis based on the

observation that its expression was found in bone marrow, spleen, and fetal liver from normal mice by RNAse probe protection and RT-PCR experiments, but not in other tissues. In particular, c-Mpl is expressed on megakaryocytes. It has also been demonstrated that the human cellular gene, human c-Mpl, is expressed in CD34 positive cells, including purified megakaryocytes and platelets. CD34 is an antigen that is indicative of early hematopoietic progenitor cells. Furthermore, exposure of CD34 positive cells to synthetic oligodeoxynucleotides that are anti-sense to the c-Mpl mRNA or message significantly inhibits the colony forming ability of CFU-MK megakaryocyte progenitors, but has no effect on erythroid or granulomacrophage progenitors.

The above data and observations suggest that c-Mpl encodes a cell surface molecule, referred to herein as the Mpl receptor, which binds to a ligand, that activates the receptor, possibly leading to production and/or development of megakaryocytes.

PCT patent publication WO 92/07074 is directed to the sequence of the protein produced by the c-Mpl gene, from both human and murine sources. This gene product, which is thought to be a receptor as explained above, is made up of at least three general regions or domains: an extracellular domain, a transmembrane domain, and an intracellular (or cytoplasmic) domain. Attached together, these domains make up the intact Mpl receptor. This PCT publication also refers to a soluble form of the receptor that substantially corresponds to the extracellular domain of the mature c-Mpl protein. The intracellular domain contains a hydrophobic region that, when attached via the transmembrane region to the extracellular domain of the protein, renders the overall protein subject to aggregation and insolubility. On the other hand, when the extracellular domain of the c-Mpl

gene product is separated from the transmembrane domain and the intracellular domain, it becomes soluble, hence the extracellular form of the protein is referred to as a "soluble" form of the receptor.

The following is a summary of some prior publications relating to the above description of the v-Mpl and c-Mpl receptors and genes:

17. Wendling, F., et al., *Leukemia* **3** (7): 475-480 (1989).

18. Wendling, F., et al., *Blood* **73** (5): 1161-1167 (1989).

19. Souyri, M., et al., *Cell* **63**: 1137-1147 (1990).

20. Vigon, I., et al., *Proc. Natl. Acad. Sci. USA* **89**: 5640-5644 (1992).

21. Skoda, R.C., et al., *The EMBO Journal* **12** (7): 2645-2653 (1993).

22. Ogawa, M., *Blood* **81** (11): 2844-2853 (1993).

23. Methia, N., et al., *Blood* **82** (5): 1395-1401 (1993).

24. Wendling, F, et al., *Blood* **80**: 246a (1993).

D. <u>The need for an agent capable of stimulating platelet production</u>.

It has been reported recently that platelet transfusions are being administered at an ever increasing rate at medical centers in North America, Western Europe, and Japan. *See* Gordon, M.S. and

Hoffman, R., *Blood* **80** (2): 302-307 (1992). This increase appears to be due in large measure to advances in medical technology and greater access to such technologies as cardiac surgery and bone marrow, heart, and liver transplantation. Dose intensification as a means of delivering therapies to cancer patients and the HIV-1 epidemic have also contributed to the heavy demand on the platelet supply.

Platelet usage carries with it the possibility of transmission of the many blood-born infectious diseases as well as alloimmunization. Moreover, the production of purified platelets is an expensive endeavor and hence the increasing use of such platelets increases overall medical costs. As a result, there exists an acute need for new and improved methods for producing platelets for human uses.

Exemplary prior approaches to enhancing platelet production are described in the following:

U.S. patent 5,032,396 reports that interleukin-7 (IL-7) is capable of stimulating platelet production. Interleukin-7 is also known as lymphopoietin-1 and is a lymphopoietic growth factor capable of stimulating growth of B- and T-cell progenitors in bone marrow. Published PCT application serial number 88/03747, filed October 19, 1988 and European patent application number 88309977.2, filed October 24, 1988 disclose DNA's, vectors, and related processes for producing mammalian IL-7 proteins by recombinant DNA technology. The data presented in the U.S. patent show that IL-7 can increase circulating platelets in normal and sublethally irradiated mice.

U.S. patent 5,087,448 discloses that megakaryocytes and platelets can be stimulated to proliferate in mammals by treating them with interleukin-6. Recombinant human interleukin-6 is a 26,000 molecular weight glycoprotein with multiple

biological activities. The data presented in this patent show that IL-6 has an effect of increasing colonies of megakaryocytes *in vitro*.

None of the above-cited patents mentions anything with respect to the Mpl ligands that are involved in the present invention.

In spite of the above disclosures, there remains a strong need for new stimulators of megakaryocytes and/or platelets in mammals.

E. <u>Background relating to Chemically Modified MGDF</u>

Proteins for therapeutic use are currently available in suitable forms in adequate quantities largely as a result of the advances in recombinant DNA technologies. Chemical derivatives of such proteins may effectively block a proteolytic enzyme from physical contact with the protein backbone itself, and thus prevent degradation. Additional advantages may include, under certain circumstances, increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. However, it should be noted that the effect of modification of a particular protein cannot be predicted. A review article describing protein modification and fusion proteins is Francis, *Focus on Growth Factors* 3: 4-10 (May 1992) (published by Mediscript, Mountview Court, Friern Barnet Lane, London N20, OLD, UK).

Polyethylene glycol ("PEG" or "peg") is one such chemical moiety which has been used in the preparation of therapeutic protein products. For example Adagen®, a formulation of pegylated adenosine deaminase is approved for treating severe combined immunodeficiency disease; pegylated superoxide dismutase has been in clinical trials for treating head injury;

pegylated alpha interferon has teen tested in phase I clinical trials for treating hepatitis; pegylated glucocerebrosidase and pegylated hemoglobin are reported to have been in preclinical testing. For some proteins, the attachment of polyethylene glycol has been shown to protect against proteolysis, Sada, et al., J. Fermentation Bioengineering **71**: 137-139 (1991), and methods for attachment of certain polyethylene glycol moieties are available. See U.S. Patent No. 4,179,337, Davis et al., "Non-Immunogenic Polypeptides," issued December 18, 1979; and U.S. Patent No. 4,002,531, Royer, "Modifying enzymes with Polyethylene Glycol and Product Produced Thereby," issued January 11, 1977. For a review, see Abuchowski et al., in Enzymes as Drugs. (J.S. Holcerberg and J. Roberts, eds. pp. 367-383 (1981)).

Other water soluble polymers have been used to modify proteins, such as copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, and poly amino acids (either homopolymers or random copolymers).

For polyethylene glycol, a variety of means have been used to attach the polyethylene glycol molecules to the protein. Generally, polyethylene glycol molecules are connected to the protein via a reactive group found on the protein. Amino groups, such as those on lysine residues or at the N-terminus, are convenient for such attachment. For example, Royer (U.S. Pat. No. 4,002,531, above) states that reductive alkylation was used for attachment of polyethylene glycol molecules to an enzyme. EP 0 539 167, published April 28, 1993, Wright, "Peg Imidates and Protein Derivates Thereof" states that peptides and organic compounds with free amino group(s) are modified with an

imidate derivative of PEG or related water-soluble organic polymers. U.S. Patent No. 4,904,584, Shaw, issued February 27, 1990, relates to the modification of the number of lysine residues in proteins for the attachment of polyethylene glycol molecules via reactive amine groups.

One specific therapeutic protein which has been chemically modified is granulocyte colony stimulating factor, "G-CSF." See European patent publications EP 0 401 384, EP 0 473, 268, and EP 0 335 423.

Another example is pegylated IL-6, EP 0 442 724, entitled, "Modified hIL-6," (see co-pending U.S.S.N. 07/632,070) which discloses polyethylene glycol molecules added to IL-6. EP 0 154 316, published September 11, 1985, reports reacting a lymphokine with an aldehyde of polyethylene glycol.

The ability to modify MGDF is unknown in the art since the susceptibility of each individual protein to modification is determined by the specific structural parameters of that protein. Moreover, the effect of such a modification on the biological properties of each protein is unpredictable from the art. Because of the many clinical applications of MGDF, as set forth herein, a derivatized MGDF product with altered properties is desirable. Such molecules may have increased half-life and/or activity *in vivo*, as well other properties.

Pegylation of protein molecules will generally result in a mixture of chemically modified protein molecules. As an illustration, protein molecules with five lysine residues and a free amino group at the N-terminus reacted in the above methods may result in a heterogeneous mixture, some having six polyethylene glycol moieties, some five, some four, some three, some two, some one and some zero. And, among the molecules with several, the polyethylene glycol moieties may not

be attached at the same location on different molecules. It will frequently be desirable to obtain a homogeneous product that contains substantially all one or a small number (e.g., 2-3) of modified protein species that vary in the number and/or location of chemical moieties, such as PEG. Nevertheless, mixtures of, e.g., mono-, di- and/or tri-pegylated species may be desirable or tolerable for a given therapeutic indication.

Variability of the mixture from lot to lot would be disadvantageous when developing a therapeutic pegylated protein product. In such development, predictability of biological activity is important. For example, it has been shown that in the case of nonselective conjugation of superoxide dismutase with polyethylene glycol, several fractions of the modified enzyme were completely inactive (P. McGoff et al. Chem. Pharm. Bull. **36**:3079-3091 (1988)). See also, Rose et al., Bioconjugate Chemistry **2**: 154-159 (1991) which reports the selective attachment of the linker group carbohydrazide to the C-terminal carboxyl group of a protein substrate (insulin). One cannot have such predictability if the therapeutic protein differs in composition from lot to lot. Some of the polyethylene glycol moieties may not be bound as stably in some locations as others, and this may result in such moieties becoming dissociated from the protein. Of course, if such moieties are randomly attached and therefore become randomly dissociated, the pharmacokinetics of the therapeutic protein cannot be precisely predictable.

Also highly desirable is a derivatized MGDF product wherein there is no linking moiety between the polymer moiety and the MGDF moiety. One problem with the above methods is that they typically require a linking moiety between the protein and the polyethylene glycol molecule. These linking moieties may be

antigenic, which is also disadvantageous when developing a therapeutic protein.

A method involving no linking group is described in Francis et al., In: "Stability of protein pharmaceuticals: in vivo pathways of degradation and strategies for protein stabilization" (Eds. Ahern., T. and Manning, M.C.) Plenum, New York, 1991) Also, Delgado et al. "Coupling of PEG to Protein By Activation with Tresyl Chloride, Applications In Immunoaffinity Cell Preparation", In: Fisher et al., ed., Separations Using Aqueous Phase Systems, Applications In Cell Biology and Biotechnology, Plenum Press, N.Y., N.Y. 1989 pp. 211-213 involves the use of tresyl chloride, which results in no linkage group between the polyethylene glycol moiety and the protein moiety. This method may be difficult to use to produce therapeutic products as the use of tresyl chloride may produce toxic by-products.

Chamow et al., Bioconjugate Chem. **5**: 133-140 (1994) report the modification of CD4 immunoadhesin with mono-methoxy-polyethylene glycol ("MePEG glycol") aldehyde via reductive alkylation. The authors report that 50% of the CD4-Ig was mePEG-modified by selective reaction at the α-amino group of the N-terminus. Id. at page 137. The authors also report that the *in vitro* binding capability of the modified CD4-Ig (to the protein gp 120) decreased at a rate correlated to the extent of MePEGylation. Ibid.

Thus, there is a need for MGDF derivatives, and, more particularly, a need for pegylated MGDF. There also exists a need for methods to carry out such derivatization.

Summary of the Invention

In one aspect, the present invention provides novel polypeptides that specifically promote megakaryocyte growth and/or development ("Mpl ligands" or "MGDFs") which are substantially free from (i.e., isolated from) other proteins (i.e., mammalian proteins in the case of an Mpl ligand obtained from a mammalian source). Such proteins may be purified from cell sources producing the factors naturally or upon induction with other factors. They may also be produced by recombinant genetic engineering techniques. Mpl ligands may further be synthesized by chemical techniques, or a combination of the above-listed techniques.

The Mpl ligands of this invention are obtainable in their native form from mammalian sources. Two exemplary Mpl ligands isolated from canine aplastic plasma are described in the examples section herein. However, it is demonstrated in other examples herein that closely related Mpl ligands are present in aplastic plasma from both human and porcine sources. Notably, the activity of each of the human, porcine, and canine Mpl ligands is specifically inhibitable by the soluble form of the murine Mpl receptor, demonstrating that all of these Mpl ligands (as well as those from other mammalian sources, including murine) are closely related both on structural and activity levels.

It is expected that human, porcine, and other mammalian Mpl ligands, may be isolated from natural sources by procedures substantially as detailed herein. *See* Example 10. Accordingly, this invention generally encompasses mammalian Mpl ligands, such as from dogs, pigs, humans, mice, horses, sheep, and rabbits. Particularly preferred Mpl ligands are those from dogs, pigs and humans.

In addition, genes encoding human Mpl ligands have been cloned from a human fetal kidney and liver libraries and sequenced, as set forth in the Example section below. Two human polypeptide sequences have been determined to have activity in a cell-based assay (see Example 4). These sequences differ in their length, but have identity over a large stretch of their amino acid sequences. The identical portions have homology to erythropoietin. The Mpl ligands are also referred to herein as Megakaryocyte Growth and Development Factors (MGDFs); all general references to Mpl ligands shall apply to those referred to herein as MGDFs and vice versa. By "MGDF polypeptide" is meant a polypeptide that has an activity to specifically stimulate or inhibit the growth and/or development of megakaryocytes. Exemplary such polypeptides are disclosed herein.

The Mpl ligands of the present invention have been found to be specifically active in the megakaryocyte lineage, augmenting maturation and/or proliferation of megakaryocytes, as demonstrated in the assays of Examples 2 and 4 below. By "specifically" is meant that the polypeptides exhibit biological activity to a relatively greater degree towards megakaryocytes as compared to many other cell types. Those that are stimulatory towards megakaryocytes are expected to have an *in vivo* activity of stimulating the production of platelets, through the stimulation of maturation and differentiation of megakaryocytes.

Two preferred Mpl ligands from a canine source have apparent molecular weights of approximately 25 kd and 31 kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Both proteins are purified during the same purification protocol which is detailed in the examples section below.

Two preferred human ligands, MGDF-1 and MGDF-2, are 332 and 173 amino acids in length, respectively, not including a 21 amino acid putative signal peptide. These sequences, and a third related molecule, MGDF-3 are shown in FIGs. 11 and 12.

Still a further aspect of the present invention are processes for isolating and purifying the Mpl ligands of the present invention or fragments thereof from mammalian sources, preferably whole blood, serum or plasma. Aplastic blood, serum or plasma are especially preferred starting materials. Aplastic blood, serum or plasma may be obtained by a process involving irradiating a mammal with a radiation source such as cobalt-60 at a radiation level of about 400-800 rads so as to render them aplastic. Such a procedure is known in the art, as exemplified in the publications cited in Example 1 below. In the case of humans, irradiated blood, plasma, or serum may be obtained from a patient after radiation therapy, e.g., to treat cancer.

Thereafter, the aplastic blood, serum or plasma is subjected to a purification process. The purification process provided by the present invention comprises the following key procedures: lectin affinity chromatography and Mpl receptor affinity chromatography. Each of these procedures results in an approximately 300-500-fold purification of the 25 and 31 kd proteins from canine aplastic plasma. Other standard protein purification procedures may be included with the above procedures to further purify the Mpl ligands of the present invention, such as those procedures detailed below.

Another aspect of the present invention includes polynucleotides that encode the expression of a mammalian Mpl ligand protein. Such DNA sequences may include an isolated DNA sequence that encodes the

expression of mammalian Mpl ligand proteins as described herein. The DNA sequences may also include 5' and 3' mammalian non-coding sequences flanking the Mpl ligand coding sequence. The DNA sequences may further encode an amino terminal signal peptide. Such sequences may be prepared by any known method, including complete or partial chemical synthesis. The codons may be optimized for expression in the host cell chosen for expression (e.g., *E. coli* or CHO cells).

Also provided by the present invention are recombinant DNA molecules, each comprising vector DNA and a DNA sequence encoding a mammalian Mpl ligand. The DNA molecules provide the Mpl ligand DNA in operative association with a regulatory sequence capable of directing the replication and expression of Mpl ligand in a selected host cell. Host cells (e.g., bacterial, mammalian insect, yeast, or plant cells) transformed with such DNA molecules for use in expressing a recombinant Mpl ligand protein are also provided by the present invention.

The DNA molecules and transformed cells of the invention are employed in another aspect, a novel process for producing recombinant mammalian Mpl ligand protein, or peptide fragments thereof. In this process a cell line transformed with a DNA sequence encoding expression of Mpl ligand protein or a fragment thereof (or a recombinant DNA molecule as described above) in operative association with a suitable regulatory or expression control sequence capable of controlling expression of the protein is cultured under appropriate conditions permitting expression of the recombinant DNA. This claimed process may employ a number of known cells as host cells for expression of the protein. Presently preferred cell lines for producing Mpl ligand are mammalian cell lines (e.g., CHO cells) and bacterial cells (e.g., *E. coli*).

For *E. coli* production of Mpl ligand, it is preferred to employ Met and Lys residues at the N-terminus of the protein to be expressed, since the yield of expression product is typically higher. A particularly preferred expression product is Met-Lys human MGDF having a total of 165 amino acids (i.e., Met^{-2}- Lys^{-1}[1-163] MGDF (numbering from the first amino acid of the mature protein). After purification of the product expressed in a bacterial cell such as *E. coli*, the terminal Met-Lys residues may be removed by treatment with an enzyme such as a dipeptidase (e.g., cathepsin C).

The expressed Mpl ligand protein is then harvested from the host cell, cell lysate or culture medium by suitable conventional means. The conditioned medium may be processed through the same purification steps or modifications thereof as used to isolate the Mpl ligand from aplastic plasma. (*See* Example 7).

In a still further aspect of the present invention, there are provided recombinant Mpl ligand proteins. These proteins are substantially free from other mammalian materials, especially proteins. The Mpl ligand proteins of this invention are also characterized by containing one or more of the physical, biochemical, pharmacological or biological activities described herein.

The present invention also relates to chemically modified MGDF comprised of a MGDF protein moiety connected to at least one water soluble polymer, and methods for the preparation and use of such compositions. In particular, the present invention includes chemically modified MGDF wherein the MGDF species is reacted with reactive polyethylene glycol molecules so as to attach PEG to MGDF. Such attachment may be accomplished by pegylation reactions discussed herein, such as acylation or alkylation. Acylation or

alkylation with PEG may be carried out under conditions whereby the major product is monopegylated or polypegylated. Polypegylation generally involves attachment of PEG to the ε-amino groups of lysine residues and may additionally involve pegylation at the N-terminus of the polypeptide. Monopegylation preferably involves attachment of PEG to the α-amino group at the N-terminus of the protein. The yield and homogeneity of such monopegylation reaction may be enhanced via a type of reductive alkylation which selectively modifies the α-amino group of the N-terminal residue of an MGDF protein moiety, thereby providing for selective attachment of a water soluble polymer moiety at the N-terminus of the protein. This provides for a substantially homogeneous preparation of polymer/MGDF protein conjugate molecules as well as (if polyethylene glycol is used) a preparation of pegylated MGDF protein molecules having the polyethylene glycol moiety directly coupled to the protein moiety.

Another aspect of this invention provides pharmaceutical compositions containing a therapeutically effective amount of isolated naturally-occurring or recombinant Mpl ligand, which may be derivatized with a water soluble polymer such as polyethylene glycol, along with a pharmaceutically acceptable carrier, diluent, or excipient. These pharmaceutical compositions may be employed in methods for treating disease states or disorders characterized by a deficiency of megakaryocytes and/or platelets as well as an *in vivo* deficiency of the Mpl ligand. They may also be employed prophylactically to ameliorate expected megakaryocyte or platelet deficiencies (e.g., due to surgery).

Thus, the Mpl ligands of the present invention may be employed in the treatment of aplastic anemias, e.g., to augment production of platelets in patients having impaired platelet production (such as AIDS

patients or patients undergoing cancer chemotherapy). Mpl ligand may be used to treat blood disorders such as thrombocytopenia. Mpl ligand may be used as an adjunctive therapy for bone marrow transplant patients. Such patients could be human or another mammal. Mpl ligand from one species is also expected to be useful in another species.

A further aspect of the invention, therefore, is a method for treating these and other pathological states resulting from a deficiency of platelets by administering to a patient a therapeutically effective amount of a pharmaceutical composition as described above. These therapeutic methods may include administration, simultaneously or sequentially with Mpl ligand, an effective amount of at least one other megakaryocyte colony stimulating factor, a cytokine (e.g., EPO), a soluble Mpl receptor, hematopoietin, interleukin, growth factor, or antibody.

Still another aspect of the present invention provides antibodies (e.g., polyclonal, monoclonal, humanized, and recombinant), and antibody fragments, directed against (i.e., reactive with) a mammalian Mpl ligand or a ligand fragment. As part of this aspect, therefore, the invention includes cells capable of secreting such antibodies (e.g., hybridomas in the case of monoclonal antibodies) and methods for their production and use in diagnostic or therapeutic procedures.

Another aspect of the invention is an assay of a body fluid for the presence of Mpl ligand. Such an assay could employ antibodies that specifically recognize an Mpl ligand, in a single antibody or "sandwich" format. Such an assay could be used to determine whether a patient needs external administration of Mpl ligand and/or whether such patient is likely to experience a platelet deficiency or

disorder. Such assays could be included in a kit format, including positive and negative controls, antibody(ies), and other standard kit components.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

<u>Brief Description of the Figures</u>

Numerous features and advantages of the present invention will become apparent upon review of the figures, wherein:

FIG. 1 depicts an overview of development and maturation of megakaryocytes and platelets.

FIG. 2 demonstrates that soluble murine Mpl receptor substantially completely inhibits the ability of plasma from irradiated dogs ("aplastic canine" or "APK9"), to induce megakaryocyte development. The assay for megakaryocyte development was that described in Example 2.

FIG. 3 shows that an activity enriched from APK9 by lectin affinity and Mpl receptor affinity chromatography procedures ("Mpl ligand") stimulates the growth of 1A6.1 cells and that soluble murine Mpl receptor blocks that growth.

FIG. 4 shows an overview of the purification steps involved in purifying the 25 and 31 kd forms of the canine Mpl receptor from aplastic canine plasma.

FIG. 5 shows the purification of Mpl ligand by reversed phase HPLC (RP-HPLC). Fraction 21 contains

highly purified 31 kd Mpl ligand; fraction 22 contains a mixture of the 31 kd and 25 kd Mpl ligands; and fraction 23 contains highly purified 25 kd Mpl ligand.

FIG. 6 shows a comparison of Mpl ligand activities in reverse phase HPLC (C4 column) fractions that contain the 25 and/or 31 kd Mpl ligand proteins.

FIG. 7 shows the number of megakaryocytes produced from cultures of CD34-selected peripheral blood cells stimulated with APK9, Mpl ligand and various other factors.

FIG. 8 shows the number of total leukocytes produced from cultures of CD34-selected peripheral blood cells stimulated with APK9, Mpl ligand and various other factors.

FIG. 9 shows the percentages of megakaryocytes that are produced in cultures of CD34-selected peripheral blood cells stimulated with APK9, Mpl ligand and various other factors.

FIG. 10 shows that human IL-3 is not involved in Mpl ligand-induced megakaryocyte development.

FIG. 11 shows the cDNA and deduced amino acid sequences of human MGDF-1 and MGDF-2.

FIG. 12 shows the cDNA and deduced amino acid sequences of human MGDF-3.

FIG. 13 shows a comparison between MGDF-1 and MGDFs (Mpl ligands) from a canine source (A) and a murine source (B).

FIG. 14 shows an example of MGDF acylation using N-hydroxysuccinimidyl (NHS) active esters of mono-methoxy-polyethylene glycol to result in a poly-pegylated product.

FIG. 15 shows an example of nonspecific MGDF reductive alkylation using mono-methoxy-polyethylene glycol aldehydes to result in a poly-pegylated product.

FIG. 16 shows an example of site-specific MGDF reductive alkylation at the α-amino group of the N-terminal residue using mono-methoxy-polyethylene glycol aldehydes to result in a substantially mono-pegylated product.

FIG. 17 shows size exclusion (SEC) HPLC analysis of MePEG-MGDF conjugates prepared using activated derivatives of MW 20kDa MePEG:

A. poly-MePEG-MGDF conjugate prepared by MGDF acylation with NHS ester of MePEG (PEG 11)

B. poly-MePEG-MGDF conjugate prepared by MGDF alkylation with MePEG aldehyde (PEG 20);

C. mono-MePEG-MGDF conjugate prepared by MGDF alkylation with MePEG aldehyde (PEG 16).

FIG. 18 shows platelet counts from mice treated with recombinant human MGDF: open diamond = CHO-derived 22-353 MGDF; open circles = unpegylated *E. coli* 22-184 MGDF (i.e., 1-163 MGDF); and closed circles = pegylated *E. coli* 22-184 MGDF.

FIG. 19 shows a purification flow chart for r-HuMGDF.

FIG. 20 shows the effect of r-HuMGDF (*E. coli* 1-163) on platelet counts in a murine carboplatin model.

Balb/c mice were intraperitoneally injected with a single dose of carboplatin (1.25 mg/mouse) at Day 0. The excipient alone group did not recieve carboplatin. After twenty-four hours, carboplatin-treated animals were subcutaneously injected with either excipient or with 100 ug/kg r-HuMGDF daily for the remainder of the study. (n=10 for each group; 5 animals were bled at every other time point).

FIG. 21 shows the effect of r-HuMGDF (*E. coli* 1-163) on platelet counts in mice treated with irradiation. Balb/c mice were irradiated with a single dose of 500 rads gamma-irradiation (Cesium source) at Day 0. The excipient alone group was not irradiated. After twenty-four hours, irradiated animals were subcutaneously injected with either excipient or with 100 ug/kg r-HuMGDF daily for the remainder of the study. (n=8 for each group; 4 animals were bled at every other time point).

FIG. 22 shows the effect of r-HuMGDF (*E. coli* 1-163) on platelet counts in mice treated with a combination of irradiation and carboplatin. Balb/c mice were irradiated with a single dose of 500 rads gamma-irradiation (Cesium source) and given carboplatin (1.25 mg/mouse) at Day 0. After twenty-four hours, the comprimised animals were subcutaneously injected with either excipient or with 100 ug/kg r-HuMGDF daily for the remainder of the study (n-8 each group). Without r-HuMGDF support, most of the animals do not survive this study. In the control group, 1 of 8 animals survived. In the treated group, 8 of 8 animals survived.

FIG. 23 shows the effect of r-HuMGDF (*E. coli* 1-163) on irradiation-induced thrombocytopenia in rhesus monkeys. Rhesus monkeys were subjected to irradiation

(700 cGy Co-80). r-HuMGDF (n=3) or human serum albumin (n=9) (each at 25 ug/kg/day) were administered subcutaneously for 18 consecutive days starting 24 hours after irradiation. Blood cell analyses were performed with an electronic blood cell analyzer. Each symbol represents the average value (+/- sem).

FIG. 24 shows the effects of pegylated and glycosylated r-HuMGDF on platelet counts in mice treated with carboplatin and irradiation. Mice were subjected to the combination of carboplatin and irradiation as described for the studies performed for FIG. 22. Subcutaneous injections of the indicated preparation of r-HuMGDF (50 ug/kg/day) were given daily for the length of the study starting 24 hours after the insult. Blood cell counts were taken on the indicated day using an electronic cell counter (Sysmex, Baxter).

FIG. 25 shows the synthetic gene sequence for recombinant human MGDF, amino acids 1-163, having *E. coli* optimized codons.

Detailed Description of the Invention

Additional aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following description, which details the practice of the invention.

The novel mammalian megakaryocyte growth promoting, and/or platelet producing factors, referred to as Mpl ligands, provided by the present invention are homogeneous proteins substantially free of association with other proteinaceous materials. Preferably, the proteins are about 90% free of other proteins, particularly preferably, about 95% free of other proteins, and most preferably about ≥98% free of other

proteins. These proteins can be produced via recombinant techniques to enable large quantity production of pure, active Mpl ligand useful for therapeutic applications. Alternatively such proteins may be obtained in a homogeneous form from mammalian aplastic blood, plasma or serum, or from a mammalian cell line secreting or expressing an Mpl ligand. Further, Mpl ligand or active fragments thereof may be chemically synthesized.

In general, by "Mpl ligands" as used in connection with the present invention is meant the Mpl ligands disclosed herein as well as active fragments and variants thereof, which are described in greater detail below.

Two preferred Mpl ligands from a canine source have apparent molecular weights of approximately 25 kd and 31 kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Both proteins are purified during the same purification protocol which is detailed in the examples section below. Thus, for example, both of these Mpl ligands bind wheat germ lectin and immobilized Mpl receptor. The 25 kd form includes an amino acid sequence as follows:

Ala-Pro-Pro-Ala-Xaa-Asp-Pro-Arg-Leu-Leu-Asn-Lys-Met-Leu-Arg-Asp-Ser-His-Val-Leu-His-Xaa-Arg-Leu-Xaa-Gln-Xaa-Pro-Asp-Ile-Tyr (SEQ ID NO: 1).

The 31 kd form includes an amino acid sequence as follows:

Ala-Pro-Pro-Ala-Xaa-Asp-Pro-Arg-Leu-Leu-Asn-Lys-Met-Leu-Arg-Asp-Ser-His-Val-Leu-His (SEQ ID NO: 2).

The "Xaa" amino acids shown in SEQ ID NOS: 1 and 2 are

not known with certainty, but are expected to be cysteine, serine, threonine, or (less likely) tryptophan.

It can be seen from the above sequences that the 31 kd ligand comprises at least a portion of the 25 kd form. In particular, the first 21 amino acids of the 31 kd protein are exactly the same as those of the 25 kd protein. This evidence, and especially the fact that both proteins have activity in the Mpl ligand activity assays presented herein, leads to the conclusion that both proteins are very closely related in terms of structure and activity. It is likely that the 31 kd form of the protein differs form the 25 kd form in differential C-terminal sequence, differential glycosylation and/or differential splicing of the gene encoding the proteins.

In addition to the above sequence information, another sequence was determined during sequencing of the 25 kd band prior to the final purification step (using reverse phase HPLC). This sequence was found associated with the 25 kd band under non-reducing conditions but not reducing conditions, implying that it is the result of cleavage into two portions (e.g., by a protease) of the 25 kd protein, which portions are held together by a disulfide bond. This sequence is:

Thr-Gln-Lys-Glu-Gln-Thr-Lys-Ala-Gln-Asp-Val-Leu-Gly-Ala-Val-Ala-Leu (SEQ ID NO: 3)

Although the precise location of SEQ ID NO: 3 in the sequence of the 25 kd protein is unclear, analogy with other cytokines, such as erythropoietin, supports the possibility that the sequence occurs around amino acid number 114 in the 25 kd protein. It should be noted that it is likely, although unproven, that SEQ ID NO: 3 also occurs in the 31 kd protein, probably again

starting around amino acid number 114. This sequence information is discussed in additional detail in Example 7.

Since the initial purification experiments of the canine ligands, summarized above, a gene encoding a canine ligand has now been cloned. As a result, the full length amino acid sequence of this canine ligand has been determined to be that set forth in FIG. 13A. Based on molecular weight calculations, it is predicted that the 25 kd and 31 kd canine ligands are C-terminal processed forms of the full-length ligand shown in FIG. 13A. Additionally, a murine Mpl ligand has been obtained having the sequence set forth in FIG. 13B.

Such purified ligands may also be characterized by specific activity in the human megakaryocyte assay of Example 2 of at least about 5.7×10^9 megakaryocyte units/mg. A megakaryocyte unit is defined as that amount of material that results in the production of as many megakaryocytes as 1 microliter of APK9 standard control using the assay described in Example 2.

Such purified ligands are also characterized by a specific activity in the Mpl-dependent cell growth assay of Example 4 of at least about 6.9×10^9 cell growth units/mg. A "cell growth unit" is defined as the amount of ligand required to result in the growth of 200 1A6.1 cells in the assay of Example 4.

The following Table 1 shows additional specific calculations of activity for actual purified canine Mpl ligands prepared in accordance with this invention:

Table 1

Mpl Ligand	1A6.1 assay (units/mg)	Human Meg assay (units/mg)
31 kd	6.52×10^9	5.7×10^9
25 kd	10.5×10^9	14×10^9

Summarizing the above information, some exemplary Mpl ligands of the present invention are characterized by one or more of the following biochemical and biological properties:

(a) such Mpl ligands are isolated from canine aplastic plasma;

(b) such Mpl ligands have apparent molecular weights of approximately 25 kd or 31 kd as determined by 12-14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions;

(c) the Mpl ligands comprise the following amino acid sequences:

SEQ ID NO: 1, in the case of the 25 kd protein; or

SEQ ID NO: 2, in the case of the 31 kd protein;

(d) the Mpl ligands additionally comprise the amino acid sequence SEQ ID NO: 3 (particularly preferably in the 25 kd protein);

(e) the Mpl ligands bind to wheat germ lectin;

(f) the Mpl ligands bind to immobilized soluble murine Mpl receptor;

(g) the Mpl ligand activity can be inhibited *in vitro* by soluble Mpl receptor; and

(h) the Mpl ligands bind to an anion exchange column at a pH of about 8-9.

The biological activities of preferred Mpl

ligands of the present invention are demonstrated by their ability to specifically stimulate the growth and development of megakaryocytes in the megakaryocyte growth promoting assay of Example 2. In this assay, MPL ligand stimulates the differentiation of human peripheral blood $CD34^+$ (i.e., CD-34 cells selected by immunoadsorption) cells during an 8 day culture period. Megakaryocytes are identified by staining with specific anti-platelet antigen antibodies and counted microscopically. MPL ligand also stimulates the growth of the factor-dependent cell line, 1A6.1. In the absence of MPL ligand, the cells will die. 1A6.1 cell number is assessed after 2 days in culture with MPL ligand.

The Mpl ligands described above have specific activities as described in Table 1 above.

Sources of the Mpl ligands have been determined to be aplastic mammalian blood, plasma, or serum. However, the source of such ligands is not expected to be limited to such known sources and may include other mammalian body fluids, cells obtained therefrom, etc.

The purification of native Mpl ligands from mammalian sources is based on two key purification steps:

(a) lectin affinity chromatography, preferably using wheat germ agglutinin; and

(b) immobilized Mpl receptor affinity chromatography.

Additional steps may be included to further purify the protein, such as ion exchange chromatography, gel filtration chromatography, and reverse phase chromatography.

The purification techniques actually employed in obtaining Mpl ligand from canine aplastic plasma comprise the following steps (*See*, Example 7):

(a) lectin affinity chromatography (wheat germ agglutinin chromatography is especially preferred);

(b) soluble Mpl receptor (Mpl-X) affinity chromatography (preferably, using immobilized murine Mpl-X);

(c) ion (anion or cation) exchange chromatography (preferably, anion exchange chromatography; particularly preferably using a Mono Q column);

(d) gel filtration chromatography under dissociative conditions (preferably, using Superdex 200 plus SDS); and

(e) reverse phase chromatography (preferably, using a C-4 column).

Homogeneous mammalian Mpl ligand, including the human ligand, may be obtained by applying the above purification procedures to aplastic blood, serum, or plasma or other sources of mammalian Mpl ligand, e.g., cell or tissue sources. The steps are not required to be in a particular sequence, but the listed sequence is preferred. Procedures for culturing a cell (or tissue) source which may be found to produce Mpl ligand are known to those of skill in the art and may be used, for example, to expand the supply of starting material.

Mpl ligand or one or more peptide fragments thereof may also be produced via recombinant techniques. To obtain the DNA sequence for a particular Mpl ligand, the purified Mpl ligand material is reduced and optionally digested with a protease such as trypsin. Enzymatic fragments are isolated and sequenced by conventional techniques. Alternatively, as exemplified in the examples herein, the intact purified protein may be sequenced directly to the extent possible based on the quantity of protein available and then the sequenced region may be used analogously to the sequenced tryptic fragments in the following procedure. Oligonucleotide

probes are synthesized using the genetic code to predict all possible sequences that encode the amino acid sequences of the sequenced fragment(s). Preferably, several degenerate sequences are generated as probes. The Mpl ligand gene is identified by using these probes to screen a mammalian genomic library or other source. Alternatively, the mRNA from a cell source of Mpl ligand can be used to make a cDNA library which can be screened with the probes to identify the cDNA encoding the Mpl ligand polypeptide. Further, the PCR technique may be used to extend the cDNA sequence, using appropriate primers.

Using these probes to screen a genomic library, a DNA clone is obtained. To obtain a full length clone, probes based on the obtained DNA sequence may be employed to rescreen the library and hybridize to the full length Mpl ligand DNA sequence.

The human cDNA for Mpl ligand can also be obtained by subcloning a full length human genomic clone into an expression vector, transfecting it into COS cells, preparing a cDNA library from these transfected COS cells and screening by hybridization for Mpl ligand cDNA. Once the entire cDNA is identified, it or any portion of it that encodes an active fragment of Mpl ligand, can be introduced into any one of a variety of expression vectors to make an expression system for Mpl ligand or one or more fragments thereof.

By such use of recombinant techniques, preferred DNA sequences encoding the Mpl ligand polypeptide are obtained. The present invention also encompasses these DNA sequences, free of association with DNA sequences encoding other proteins (i.e., isolated), and coding for expression of Mpl ligand polypeptides with an Mpl ligand activity (e.g., megakaryocyte growth and/or development). These DNA sequences include those sequences encoding all or a

fragment of Mpl ligand and those sequences which hybridize, preferably under stringent hybridization conditions to the cDNA sequences [*See*, T. Maniatis et. al., *Molecular Cloning* (A Laboratory Manual); Cold Spring Harbor Laboratory (1982), pages 387 to 389].

Exemplary stringent hybridization conditions are hybridization in 4 X SSC at 62-67° C., followed by washing in 0.1 X SSC at 62-67° C. for approximately an hour. Alternatively, exemplary stringent hybridization conditions are hybridization in 45-55% formamide, 4 X SSC at 40-45°C.

DNA sequences which hybridize to the sequences for Mpl ligand under relaxed hybridization conditions and which encode Mpl ligand peptides having Mpl ligand biological properties also encode novel Mpl ligand polypeptides of this invention. Examples of such relaxed stringency hybridization conditions are 4 X SSC at 45-55°C. or hybridization with 30-40% formamide at 40-45° C. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation or disulfide linkages, with the sequences of Mpl ligand and encodes a protein having one or more Mpl ligand biological properties clearly encodes an Mpl ligand polypeptide even if such a DNA sequence would not stringently hybridize to the Mpl ligand sequence(s).

Allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) of DNA sequences encoding the peptide sequences of Mpl ligand are also included in the present invention, as well as analogs or derivatives thereof. Similarly, DNA sequences which code for Mpl ligand polypeptides but which differ in codon usage due to the degeneracies of the genetic code or variations in the DNA sequence of Mpl ligand which are caused by point mutations or by induced modifications to enhance the activity, half-life or

production of the polypeptides encoded thereby are also encompassed in the invention.

A cloning procedure as set forth in Example 11 below was followed and resulted in the amino acid and cDNA sequences of the human proteins MGDF-1, MGDF-2, and MGDF-3 disclosed herein. MGDF-1 is shown as amino acids 22-353 in FIG. 11 and contains 332 amino acids. MGDF-2 is a truncated portion of MGDF-1, and is shown as amino acids 22-195 in FIG. 11. MGDF-2 therefore contains 174 amino acids. MGDF-3 is shown as amino acids 22-289 in FIG. 12 and contains 268 amino acids. In each MGDF disclosed herein, the molecule including the signal peptide, shown as amino acids 1-21 in both FIGS. 11 and 12, is also part of the present inventive polypeptides, but it is preferably removed for megakaryocyte growth and development activity to be exhibited. In summary, MGDFs 1-3 are defined as follows:

MGDF-1	amino acids	22-353	FIG. 11
MGDF-2	amino acids	22-195	FIG. 11
MGDF-3	amino acids	22-289	FIG. 12.

In the assays presented herein, MGDF-1 and MGDF-2 were active whereas MGDF-3 was not.

Based on the activity data presented herein, it is hypothesized that human MGDF is expressed *in vivo* as a substantially inactive or less active precursor polypeptide that contains variable C-terminal amino acids. Upon cleavage of the C-terminal amino acids (as well as the signal peptide), the processed form(s) of the molecule retain activity or become more active. In view of the above hypothesis, it is believed that MGDF-1 may require processing (e.g., cleavage with a protease) in order to exhibit its activity. The fact that a truncated form of MGDF-1 (i.e., MGDF-2) is active supports this hypothesis.

Conditioned medium from human kidney 293 cells (Invitrogen) transfected with the MGDF-1 gene does demonstrate activity in the cell assay of Example 4 below. On the other hand, in other cell lines, e.g., 32 D cells, no activity was seen for this molecule. It is hypothesized that this may mean that 293 cells are able to process the MGDF-1 molecule, presumably by truncation, so that the molecule primarily responsible for the activity is a truncated form, whereas the 32 D cells are unable to process the molecule.

In view of the above hypothesis, various active molecules may result from truncations of the sequence set forth as MGDF-1 (FIG. 11). Structural features conserved among the cytokine family, such as erythropoietin (EPO), include four α-helical bundles and four cysteines. Referring to the MGDF-1 sequence, Cys 172 is the most C-terminal element of these evolutionarily conserved and functionally essential structures. Therefore, preferred truncation variants of MGDF-1 are any of those that have C-terminal truncations from amino acid 173 to 353 (along with cleavage of the signal peptide). Preferably, the sequence of MGDF-1 will have removed from it from 50 to 185 amino acids from the C-terminus, particularly preferably, from 90 to 172 amino acids from the C terminal. As disclosed herein, the signal peptide is thought to be 21 amino acids in length; however, the signal peptide may have 23 amino acids, based on the sequence of MGDF-1. Accordingly, polypeptides corresponding to those presented herein but which start at position 24 of FIG. 11 or 12 are also specifically contemplated.

The following are some specific preferred variants of MGDF-1 that may exhibit activity (i.e., the ability to promote the growth of megakaryocytes and/or platelets; or inhibitory/ stimulatory activity towards the natural receptor):

MGDF-4	amino acids	22-172	FIG. 11
MGDF-5	amino acids	22-177	FIG. 11
MGDF-6	amino acids	22-191	FIG. 11
MGDF-7	amino acids	22-198	FIG. 11
MGDF-8	amino acids	22-265	FIG. 11
MGDF-11	amino acids	22-184	FIG. 11

In some clones, amino acids 133-136 in the MGDF-1 sequence were absent, so that sequences corresponding to those set forth above, but in which these amino acids are missing (and the C-terminus amino acid number adjusted down by 4) may also be active.

In one clone, which had a termination codon at position 192, an Ala residue was found instead of a Thr residue as shown at position 191 in FIG. 11. Therefore, the invention includes variants of MGDF molecules in which position 191 is Ala instead of Thr.

MGDF-3 results from removal of a sequence referred to herein as IVS-5 (Intervening Sequence-5) since this sequence is spliced within the fifth exon in the sequence. Since the 5' end of IVS-5 occurs within a codon, its removal results in a frame-shift in the remaining sequence of MGDF, which can be seen to occur starting at position 160 of MGDF-3 to the end of the molecule.

No activity has yet been found for MGDF-3 itself upon transfection into 293 cells and testing the resulting conditioned medium for activity in the cell-based assay of Example 4. Apparently, unlike MGDF-1, 293 cells are unable to process MGDF-3 to an active form. Nevertheless, based on the truncation hypothesis set forth above in connection with MGDF-1, truncation of C-terminal amino acids from MGDF-3 may also result in activity. For example, C-terminal truncation of MGDF-3 of from 40 to 102 amino acids may result in activity.

Preferably, from 50 to 90 amino acids are removed. Two specific preferred variants of MGDF-2 are:

MGDF-9	22-179	FIG. 12
MGDF-10	22-190	FIG. 12

In all of the Mpl ligands disclosed herein, including the exemplary MGDFs set forth above, a methionyl residue may be present at the N-terminus, especially when such polypeptides are expressed in bacterial host cells.

Mpl ligand polypeptides may also be produced by known conventional chemical synthesis. Methods for constructing the polypeptides of the present invention by synthetic means are known to those of skill in the art. The synthetically-constructed Mpl ligand polypeptide sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with Mpl ligand polypeptides may possess Mpl ligand biological properties in common therewith. Thus, they may be employed as biologically active or immunological substitutes for natural, purified Mpl ligand polypeptides in therapeutic and immunological processes.

Modifications in the peptides or DNA sequences encoding Mpl ligand can be made by one skilled in the art using known techniques. Modifications of interest in the Mpl ligand sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences. Mutagenesis techniques for such replacement, insertion or deletion are well known to one skilled in the art. [*See*, e.g., U.S. Pat. No. 4,518,584.] Conservative changes in from 1 to 20 amino acids are preferred. Preferred peptides may be generated by proteolytic enzymes, or by direct chemical synthesis. Such variants are included within the

meaning of Mpl ligand polypeptides and polynucleotides of the present invention.

Specific mutations of the sequences of the Mpl ligand polypeptide may involve modifications of a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Expression of such altered nucleotide sequences produces variants which are not glycosylated at that site.

Additional Analogs/Derivatives of MGDF

Other analogs and derivatives of the sequences of MGDF (Mpl ligands), which may retain MGDF (Mpl ligand) activity in whole or in part may also be prepared by one of skill in the art given the disclosures herein. Such modifications are also encompassed by this invention.

More particularly, the present invention also broadly includes chemically modified MGDF compositions and methods of making and using them. The present

disclosure reveals that it is possible to modify MGDF and to enhance its properties.

In one aspect, the present invention relates to an MGDF product comprising an MGDF protein linked to at least one water soluble polymer moiety.

In another aspect, the present invention relates to an MGDF product wherein said MGDF protein is linked to at least one polyethylene glycol molecule.

In another aspect, the present invention relates to MGDF molecules attached to at least one polyethylene glycol molecule via an acyl or alkyl linkage.

Pegylation of MGDF may be carried out by any of the pegylation reactions known in the art. See, for example: *Focus on Growth Factors* 3 (2): 4-10 (1992); EP 0 154 316; EP 0 401 384; and the other publications cited herein that relate to pegylation. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). These preferred means for derivatization with polyethylene glycol will now be discussed in greater detail.

Acylation

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with an MGDF protein. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of MGDF. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide ("NHS"). As used herein, "acylation" is contemplated to included without limitation the following types of linkages between MGDF and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like. See Bioconjugate Chem. **5**: 133-140 (1994).

Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions such as temperature, solvent, and pH that would inactivate the MGDF species to be modified. Reaction conditions that apply generally to pegylation of MGDFs will be described below. An exemplary reaction with an NHS ester of monomethoxy-PEG is depicted in FIG. 14.

Pegylation by acylation will generally result in a poly-pegylated MGDF product, wherein the lysine ε-amino groups are pegylated via an acyl linking group. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., ≥ 95%) mono, di- or tri-pegylated. However, some species with higher degrees of pegylation (up to the maximum number of lysine ε-amino acid groups of MGDF plus one α-amino group at the amino terminus of MGDF) will normally be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Alkylation

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with a protein such as MGDF in the presence of a reducing agent. As with acylation, discussed above, the reaction conditions are described below.

Pegylation by alkylation can also result in poly-pegylated MGDF. An exemplary reductive alkylation reaction with MGDF to yield a polypegylated product is shown in FIG. 15. In addition, one can manipulate the

reaction conditions as described herein to favor pegylation substantially only at the α-amino group of the N-terminus of the MGDF species (i.e., a mono-pegylated species). An exemplary reductive alkylation reaction with MGDF to yield a monopegylated product is shown in FIG. 16. In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a $-CH_2-NH-$ group. With particular reference to the $-CH_2-$ group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in MGDF. The reaction is performed at a pH (see below) which allows one to take advantage of the pK_a differences between the ε-amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. In one important aspect, the present invention provides for a substantially homogeneous preparation of monopolymer/MGDF protein conjugate molecules (meaning MGDF protein to which a polymer molecule has been attached substantially only (i.e., ≥ 95%) in a single location). More specifically, if polyethylene glycol is used, the present invention also provides for pegylated MGDF protein lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the MGDF protein.

Thus, in a preferred aspect, the present

invention relates to pegylated MGDF, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, preferably 2-5, PEG groups). The PEG groups are generally attached to the protein at the α or ε amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

The polymer molecules used in both the acylation and alkylation approaches may be selected from among water soluble polymers or a mixture thereof. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, preferably, so that the degree of polymerization may be controlled as provided for in the present methods. A preferred reactive PEG aldehye is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see, U.S. Patent 5,252,714). The polymer may be branched or unbranched. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, monomethoxy-polyethylene glycol, dextran, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive

alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally-occurring glycosyl residues since these are usually made more conveniently by mammalian recombinant expression systems. The polymer may be of any molecular weight, and may be branched or unbranched.

A particularly preferred water-soluble polymer for use herein is polyethylene glycol, abbreviated PEG. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

As employed herein, MGDF is defined as including any of the various forms of MGDF described herein. For example, full-length or truncated, glycosylated or nonglycosylated forms of MGDF are all included. The following are preferred MGDF molecules to be derivatized (in each case the numbering refers to the amino acids numbered in accordance with FIG. 11):

MGDF-1	amino acids	22-353	FIG. 11
MGDF-2	amino acids	22-195	FIG. 11
MGDF-4	amino acids	22-172	FIG. 11
MGDF-11	amino acids	22-184	FIG. 11
MGDF-12	amino acids	27-353	FIG. 11
MGDF-13	amino acids	27-195	FIG. 11
MGDF-14	amino acids	27-172	FIG. 11
MGDF-15	amino acids	27-184	FIG. 11

The above-preferred species may be glycosylated, non-glycosylated, or de-glycosylated, preferably non-glycosylated. They may be made recombinantly in either bacterial (e.g., *E. coli*) or mammalian (e.g., CHO) cells.

The following are particularly preferred sub-

groups of chemically derivatized molecules of this invention (in each case, they are mono- or poly-, e.g., 2-4, PEG moieties, attached via an acyl or alkyl group):

pegylated MGDF-11
pegylated MGDF-4
pegylated MGDF-2.

In general, chemical derivatization may be performed under any suitable condition used to react a biologically active substance with an activated polymer molecule. Methods for preparing pegylated MGDF will generally comprise the steps of (a) reacting an MGDF polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby MGDF becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of PEG: protein, the greater the percentage of poly-pegylated product.

Reductive alkylation to produce a substantially homogeneous population of mono-polymer/MGDF protein conjugate molecule will generally comprise the steps of: (a) reacting an MGDF protein with a reactive PEG molecule under reductive alkylation conditions, at a pH suitable to permit selective modification of the α-amino group at the amino terminus of said MGDF protein; and (b) obtaining the reaction product(s).

For a substantially homogeneous population of mono-polymer/MGDF protein conjugate molecules, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of MGDF. Such reaction

conditions generally provide for pK_a differences between the lysine amino groups and the α-amino group at the N-terminus (the pK_a being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal α-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-9, preferably 3-6.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2kDa to about 100kDa (the term "about" indicating ± 1kDa). The preferred average molecular weight is about 5kDa to about 50kDa, particularly preferably about 12kDa to about 25kDa. The ratio of water-soluble polymer to MGDF protein will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any MGDF protein having an α-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/MGDF

protein conjugate. The term "monopolymer/MGDF protein conjugate" is used here to mean a composition comprised of a single polymer molecule attached to an MGDF protein molecule. The monopolymer/MGDF protein conjugate preferably will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will preferably be greater than 90% monopolymer/MGDF protein conjugate, and more preferably greater than 95% monopolymer MGDF protein conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety). The examples below provide for a preparation which is at least about 90% monopolymer/ protein conjugate, and about 10% unreacted protein. The monopolymer/protein conjugate has biological activity.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents may be selected from the group consisting of sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly preferred reducing agent is sodium cyanoborohydride.

Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined case-by-case based on the published information relating to derivatization of proteins with water soluble polymers (see the publications cited herein). Exemplary details are shown in the Examples section below.

One may choose to prepare a mixture of polymer/protein conjugate molecules by acylation and/or alkylation methods, and the advantage provided herein is that one may select the proportion of monopolymer/ protein conjugate to include in the mixture. Thus, if

desired, one may prepare a mixture of various protein with various numbers of polymer molecules attached (i.e., di-, tri-, tetra-, etc.) and combine with the monopolymer/protein conjugate material prepared using the present methods, and have a mixture with a predetermined proportion of monopolymer/protein conjugate.

The working examples below demonstrate the preparation of chemically modified MGDF and the preparation of MGDF pegylated via acylation and alkylation. Thus, other aspects of the present invention relate to these preparations.

Generally, conditions which may be alleviated or modulated by administration of the present polymer/MGDF include those described above for MGDF molecules in general. However, the polymer/MGDF molecules disclosed herein may have additional activities, enhanced or reduced activities, or other characteristics, as compared to the non-derivatized molecules.

In yet another aspect of the present invention, provided are pharmaceutical compositions of the above chemically modified MGDF molecules. Such pharmaceutical compositions may contain any of the ingredients specified herein for non-derivatized MGDF molecules.

As further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain proper dosing. Generally, the dosage will be between 0.01 μg/kg body weight (calculating the mass of the protein alone, without chemical modification) and 300 μg/kg (based on the same). The preferred dose will generally be from 5

μg/kg body weight to 100 μg/kg body weight, particularly preferably from 10 μg/kg body weight to 75 μg/kg body weight.

The present invention also provides a method for producing MGDF (i.e., Mpl ligand) polypeptides or active fragments thereof. One method of the present invention involves introducing the cDNA encoding an Mpl ligand polypeptide into an expression vector to make an expression system for Mpl ligand. A selected host cell is transfected with the vector and cultured. The method of this present invention therefore comprises culturing a suitable cell or cell line, which has been transfected with a DNA sequence coding on expression for an Mpl ligand polypeptide under the control of known regulatory sequences. Regulatory sequences include promoter fragments, terminator fragments and other suitable sequences which direct/control the expression of the protein in an appropriate host cell. The expressed factor is then recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by appropriate means known to one of skill in the art. Additionally, the methods disclosed in U.S. Patent 5,272,071 are also contemplated to be applicable to the inventive polynucleotides/ polypeptides.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. *See*, e.g., Gething and Sambrook, *Nature* **293**: 620-625 (1981), or alternatively, Kaufman et al., *Mol. Cell. Biol.*, **5** (7): 1750-1759 (1985) or Howley et al., *U.S. Pat. No. 4,419,446*. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7

cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5α, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al., *Genetic Engineering* **8**: 277-298 (1986) and references cited therein.

The present invention also provides recombinant molecules or vectors for use in the method of expression of novel Mpl ligand polypeptides. These vectors contain the Mpl ligand DNA sequences and which alone or in combination with other sequences code for Mpl ligand polypeptides (with or without signal peptides) of the invention or active fragments thereof. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of Mpl ligand polypeptides. The vector employed in the method also

contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

One vector is pXM, which is particularly desirable for expression in COS cells [Y.C. Yang et al., *Cell* **47**: 3-10 (1986)]. Another vector which is desirable for expression in mammalian cells, e.g., CHO cells, is pEMC2B1. Mammalian cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors, e.g. replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures. *See*, Kaufman et al., *J. Mol. Biol.* **159**: 511-521 (1982); and Kaufman, *Proc. Natl. Acad. Sci. USA* **82**: 689-693 (1985). Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome [Lusky et al., *Cell* **36**: 391-401 (1984)] and be replicated in cell lines such as C127 mouse cells as a stable episomal element. The transfection of these vectors into appropriate host cells can result in expression of the Mpl ligand polypeptides.

Other appropriate expression vectors of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression can also be used for this purpose.

The conditions to be treated by the methods and compositions of the present invention are generally those which involve an existing megakaryocyte/platelet deficiency or an expected megakaryocyte/platelet deficiency in the future (e.g., because of planned surgery). Such conditions will usually be the result of a deficiency (temporary or permanent) of active Mpl ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods

and compositions of the present invention are generally available for treating thrombocytopenia.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions. Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic tumors which result in thrombocytopenia, systemic lupus erythematosus, splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery, an Mpl ligand of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, an Mpl ligand could be administered along with blood or purified platelets.

The Mpl ligands of this invention may also be useful in stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that express the Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

MGDF molecules that are not themselves active in the activity assays presented herein may be useful as modulators (e.g., inhibitors or stimulants) of the Mpl receptors *in vitro* or *in vivo*.

The polypeptides of the present invention may also be employed alone, or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies, in the treatment of the above-identified conditions.

Therefore, as yet another aspect of the invention are therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of an Mpl ligand polypeptide or a therapeutically effective fragment thereof in admixture with a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably supplemented with other materials common in solutions for administration to mammals. Typically, an Mpl ligand therapeutic will be administered in the form of a composition comprising purified protein in conjunction with one or more physiologically acceptable carriers, excipients, or diluents. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (e.g., sucrose). Other standard carriers, diluents, and excipients may be included as desired. Other exemplary compositions are Tris buffer, pH 8.0 and acetate buffer, pH 5.0, which, in each case, may further include sorbitol.

The present compositions can be systemically administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 0.1-1000 micrograms of Mpl ligand protein or fragment thereof per kilogram of body weight.

The therapeutic methods, compositions and polypeptides of the present invention may also be employed, alone or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that an Mpl ligand molecule will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with Mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), IFN-beta, or IFN-gamma. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus,

administration of Mpl ligand (to enhance the number of mature megakaryocytes) followed by administration of the soluble Mpl receptor (to inactivate the ligand and allow the mature megakaryocytes to produce platelets) is expected to be a particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

Other uses for these novel polypeptides are in the development of antibodies generated by standard methods. Thus, antibodies that react with the Mpl ligands of the present invention, as well as reactive fragments of such antibodies, are also contemplated. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific, etc. The antibody fragments may be any fragment that is reactive with the Mpl ligand of the present invention, such as, F_{ab}, $F_{ab'}$, etc. Also provided by this invention are the hybridomas generated by presenting Mpl ligand or a fragment thereof as an antigen to a selected mammal, followed by fusing cells (e.g., spleen cells) of the animal with certain cancer cells to create immortalized cell lines by known techniques. The methods employed to generate such cell lines and antibodies directed against all or portions of a human Mpl ligand polypeptide of the present invention are also encompassed by this invention.

The antibodies may be used therapeutically, such as to inhibit binding of the Mpl ligand and its receptor. The antibodies may further be used for *in vivo* and *in vitro* diagnostic purposes, such as in labeled form to detect the presence of the Mpl ligand in a body fluid.

* * *

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention, but are not meant to limit the scope thereof, unless so indicated. Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, Sambrook et al., *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press (1987) and in Ausubel et al., (Eds), *Current Protocols in Molecular Biology*, Greene associates/Wiley Interscience, New York (1990).

EXAMPLE 1

Aplastic Canine Plasma

Heparinized aplastic canine plasma ("APK9") or normal canine plasma ("NK9") was produced as described in the following publications, except that 450 rads of total body irradiation were delivered to recipients:

1. Mazur, E. and South, K. *Exp. Hematol.* **13**:1164-1172 (1985).

2. Arriaga, M., South, K., Cohen, J.L., and Mazur, E.M. *Blood* **69**: 486-492 (1987).

3. Mazur, E., Basilico, D., Newton, J.L., Cohen, J.L., Charland, C., Sohl, P.A., and Narendran, A. *Blood* **76**: 1771-1782 (1990).

EXAMPLE 2

Human Megakaryocyte Assay

APK9 and fractionated APK9 were assayed for the ability to stimulate development of human megakaryocytes from $CD34^+$ progenitor cells. CD34-selected cells were obtained from peripheral blood cells as described (Hokom, M.H., Choi, E., Nichol, J.L., Hornkohl, A., Arakawa, T., and Hunt, P. *Molecular Biology of Haematopoiesis* 3:15-31,1994) and were incubated in the following culture medium: Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 1% Glutamine Pen-strep (Irvine Scientific, Santa Ana, CA) and 10% heparinized, platelet-poor, human AB plasma. Also included were 2-mercaptoethanol (10^{-4} M), pyruvic acid (110 µg/ml), cholesterol (7.8 µg/ml), adenosine, guanine, cytidine, uridine, thymidine, 2-deoxycytosine, 2-deoxyadenosine, 2-deoxyguanosine (10 µg/ml each, Sigma); human recombinant insulin (10 µg/ml), human transferrin (300 µg/ml), soybean lipids (1%, Boehringer Mannheim, Indianapolis, IN); human recombinant basic fibroblast growth factor (2 ng/ml, Genzyme, Cambridge, MA); human recombinant epidermal growth factor (15 ng/ml), platelet-derived growth factor (10 ng/ml, Amgen, Inc., Thousand Oaks, CA). CD34-selected cells were plated at $2x10^5$/ml culture medium, 15 ul final volume, in wells of Terasaki-style microtiter plates (Vanguard, Inc., Neptune, NJ). Cells were incubated at 37°C for 8 days in humidified boxes in 5% CO_2 in air, fixed directly to the culture wells with 1% glutaraldehyde, and incubated with a monoclonal antibody cocktail (anti-GPIb, anti-GPIIb, (Biodesign) and anti-GPIb (Dako, Carpinteria, CA). The immune reaction was developed with a streptavidin-beta-galactosidase detection system

(HistoMark, Kirkegaard and Perry). Megakaryocytes, identified by a blue color, were counted with an inverted phase microscope at 100X magnification. Results were presented as the average number of megakaryocytes per well +/- standard error of the mean (SEM). In some cases, data were presented in terms of "megakaryocyte units/ml" where the degree to which a given sample induced megakaryocyte development was normalized to the positive APK9 control for that experiment. One unit is defined as the amount of material that results in the same number of megakaryocytes as 1 ul of APK9 standard. Activity was accepted as due to MPL ligand if it could be blocked with 5-10 ug/ml MPL-X (soluble Mpl receptor).

APK9 has been demonstrated to contain factor(s) that stimulate human megakaryocyte development in this system. CD34-selected cells incubated with 10% NK9 for 8 days show a negligible number of blue-stained megakaryocytes, whereas CD34-selected cells incubated with 10% APK9 for 8 days show a very large number of blue-stained megakaryocytes.

FIG. 2 shows that increasing concentrations of Mpl-X added to the human megakaryocyte culture system increasingly block megakaryocyte development. At concentrations of Mpl-X greater than 5 µg/ml, inhibition is complete. In this experiment, CD34-selected cells were stimulated with 5% APK9. This demonstrates that an activity which interacts with Mpl-X (presumptive Mpl ligand) is necessary for human megakaryocyte development, and implies that the Mpl ligand is present in APK9 itself.

It has been further demonstrated herein that the Mpl ligand activity necessary for human megakaryocyte development is present in APK9. APK9 (135 ml) was diluted 6-fold into Iscove's media and applied to an Mpl-X affinity column. Unbound material (flow

through) was collected and concentrated to the original volume before assay. Bound material was eluted in 10 ml of 1 M NaCl, and 20% of the pool was diafiltered and concentrated 4-fold for assay. CD34-selected cells incubated in media alone did not develop into megakaryocytes. Cells incubated in 5% APK9 (same pool as applied to column) developed into 48 +/- 8 megakaryocytes per well. Cells incubated in 10% of the unbound material did not develop into megakaryocytes. Cells incubated in 10% of the elution pool developed into 120 +/- 44 megakaryocytes per well. Both the column load and the elution pool activities were substantially completely inhibited with 5 µg/ml Mpl-X in the assay.

EXAMPLE 3

Transfection of murine or human Mpl receptor into a murine cell line

A. Murine Mpl Receptor

The full length murine Mpl receptor cDNA was subcloned into an expression vector containing a transcriptional promoter derived from the LTR of Moloney Murine Sarcoma virus. 5 µg of this construct and 1 µg of the selectable marker plasmid pWLNeo (Stratagene) were co-electroporated into an IL-3 dependent murine cell line (32D, clone 23; Greenberger et al., *PNAS* 80:2931-2936 (1983)). Cells were cultured for 5 days to recover from the procedure, then incubated in selection media including 800 ug/ml Geneticin (G418, Sigma) and 1 ng/ml murine IL-3. The surviving cells were then divided into pools of $2x10^5$ cells and cultured until a population grew out which could be further analyzed. Six populations were tested for surface expression of Mpl receptor by FACS analysis using a polyclonal rabbit

antipeptide serum. One population was chosen for FACS sorting using the same antipeptide serum as before. Single-cell clones of the parent cell line were selected by growth in 10% APK9 and Geneticin. After selection in APK9 for 35 days, the cells were maintained in 1 ng/ml murine IL-3. One of the subclones, 1A6.1, was used for this body of work.

B. Human Mpl Receptor

The full length human Mpl receptor sequence (Vigon, I., et al., *PNAS* **89**: 5640-5644 (1992)) was subcloned into an expression vector containing the transcriptional promoter of Moloney Murine Sarcoma virus (same vector as with the murine receptor). Six µg of this construct and 6 µg of an amphotrophic retroviral packaging construct (Landau, N.R., Littman, D.R., *J. Virology* **66**: 5110-5113 (1992)) were transfected into 3×10^6 293 cells using a $CaPO_4$ mammalian transfection kit (Stratagene). The same cells were retransfected after 2 days and again after 4 days. The day after the last transfection the 293 cells were cocultivated with the IL-3 dependent murine cell line (32D, clone 23; Greenberger et al., *PNAS* **80**: 2931-2936 (1983)). After 24 hours, the 32D cells were rescued and banded in a BSA gradient (Path-o-cyte; Miles Inc.). Cells were expanded in 1 ng/ml murine IL-3 and then were selected for growth in 20% APK9. Cells were sorted for cell surface expression of receptor by FACS using a polyclonal rabbit antipeptide serum. These cells were subsequently used in the assays.

EXAMPLE 4

1A6.1 assay for Mpl ligand

1A6.1 cells were washed free of culture IL-3 and replated (1000 cells/15 µl total vol/ well) in Terasaki-style microtiter plates in alpha MEM (Gibco) supplemented with 10% fetal calf serum (FCS), Geneticin (800 µg/ml) and 1% pen/strep (Gibco) in 1:1 serial dilutions of test samples. After 48 hours, the number of viable cells per well was determined microscopically. One unit of activity was defined as that amount of activity that resulted in 200 viable cells per well. Activity was defined as due to Mpl ligand if it could be completely blocked by including 5-10 µg/ml Mpl-X in the assay. Mpl ligand activity in APK9 averaged 4400 +/- 539 units/ml of aplastic plasma. Unless otherwise indicated, units of Mpl ligand activity are defined in the 1A6.1 assay.

Assays with cells transfected with the human Mpl receptor gene (Example 3B) were carried out in essentially the same manner as with the 1A6.1 cells.

EXAMPLE 5

Demonstration that Mpl-ligand is present in aplastic plasma or sera of mouse, dog, pig and human sources

Mpl ligand is present in the aplastic plasma or sera from murine, canine, porcine and human sources (Table 2). Plasma was collected from BDF1 mice pre-irradiation and 12 days post-irradiation (500 rads). Plasma was tested in the 1A6.1 assay where it demonstrated 2000 units/ml activity that was substantially completely inhibitable with Mpl-X (10

ug/ml). Irradiated mouse plasma was also positive in the human megakaryocyte assay where it displayed an activity of 1833 units/ml. Plasma was collected from dogs pre-irradiation and 10 days post-irradiation (450 rads). Plasma was tested in both the 1A6.1 assay and human megakaryocyte assays. Activity was detected and completely inhibited by Mpl-X (10 ug/ml) in both assays. Plasma was collected from pigs pre-irradiation and 10 days post-irradiation (650 rads). Plasma was tested in both the 1A6.1 assay and the human megakaryocyte assays. In both assays it displayed Mpl ligand activity (inhibitable by 10 ug/ml Mpl-X) comparable to that found in aplastic canine plasma. Sera from aplastic humans was obtained. This material was collected from bone marrow transplantation patients. The sera from 6 patients were assayed in the 1A6.1 assay where it showed an activity of 903 units/ml, 88% of which was due to Mpl ligand (inhibitable with 10 ug/ml Mpl-X). Sera from 14 aplastic patients has also been tested in the human megakaryocyte assay. As a group, they displayed substantial activity, 941 meg units/ml, which was completely inhibitable with 10 ug/ml Mpl-X. Murine IL-3 data is included to demonstrate the specificity of the 1A6.1 assay. Although this recombinant cytokine induces growth of the cell line, it is not blocked by 10 ug/ml Mpl-X.

Table 2

Species	1A6.1 Cell Assay (units/ml)		Human Meg Assay (meg units/ml)	
	media	+ Mpl-X	media	+Mpl-X
Normal mouse	0+/-0	0+/-0	0+/-0	0+/-0
Aplastic mouse	2000	0	1833	not done
Normal canine	0+/-0	0+/-0	0+/-0	0+/-0
Aplastic canine	4400+/-539	0+/-0	792+/-128	0+/-0
Normal porcine	0+/-0	0+/-0	0+/-0	0+/-1
Aplastic porcine	3866+/-1136	0+/-0	1284+/-182	10+/-10
Normal human	0+/-0	0+/-0	0+/-0	0+/-0
Aplastic human	903+/-64	114+/-33	941+/-178	0+/-0
murIL3	6000+/-565	6000+/-565	not done	not done

EXAMPLE 6

Mpl ligand stimulates 1A6.1 cell growth and human megakaryocyte development

Mpl ligand (enriched at least about 100,000-fold after lectin and affinity chromatography procedures; *see* Example 7) stimulates the growth of the 1A6.1 cell line and the development of human megakaryocytes from CD34-selected peripheral blood cells in a dose-dependent manner. The activity responsible is due to Mpl ligand as shown in FIGS. 2 and 3 since the activities in both assays can be completely blocked with Mpl-X.

It has also been shown by the inventors that FACS purified peripheral blood $CD34^+$ cells, when

incubated in Mpl ligand (100 units/ml for 9 days in this case), develop into phenotypically normal, mature megakaryocytes. This establishes that purified Mpl ligand has the same effect on megakaryocytes as does crude APK9. Furthermore, this experiment used purified $CD34^+$ cells (100% $CD34^+$) as opposed to CD34-selected cells which are generally only 30-50% $CD34^+$.

EXAMPLE 7

Purification of Canine Mpl Ligand

I. Summary

Proteins (25 kd and 31 kd) that display activities predicted for a ligand for the Mpl receptor were purified. The proteins were purified from the plasma of irradiated dogs by a scheme employing wheat germ agglutinin (WGA) affinity chromatography, Mpl receptor affinity chromatography, anion exchange chromatography, gel filtration chromatography, and C4 reversed phase HPLC. *See*, FIG. 4 for an overview of this purification scheme. The 25 kd and 31 kd Mpl ligands have been highly purified to apparent homogeneity and have been determined to contain the amino acid sequences disclosed herein.

II. Methods

A. Clarification of plasma.

Frozen plasma (a total of 20 liters) from irradiated dogs (*see* Example 1) was thawed overnight at 4°C; thawing of larger bottles was initiated at room temperature for several hours before placement in the cold room. Insoluble material was removed by centrifugation for 6 hours at 11,000 xg. The plasma was

diluted with phosphate buffered saline, pH 7.3, containing 0.01% sodium azide (PBS/azide) and filtered through a 1.2 µm filter. The clarification procedure typically resulted in an approximate two-fold dilution of the starting material.

B. Wheat Germ Agglutinin Affinity Chromatography.

All operations were carried out at 4°C. The clarified plasma (in two batches) was applied to a column of immobilized wheat germ agglutinin (1 liter, 10 x 12 cm, E Y Laboratories), equilibrated in PBS/azide. After sample application, unbound material was washed from the column with PBS/azide, followed by a wash with 0.5 M NaCl in 20 mM Tris-HCl, pH 8. Mpl ligand activity, bound by the WGA column, was eluted with 0.35 M N-acetylglucosamine (GlcNAc), 0.5 M NaCl, 20 mM Tris-HCl, pH 8. Mpl ligand activity could not be detected in the flow through or wash fractions.

C. Mpl-X receptor affinity chromatography.

The soluble murine Mpl receptor (Mpl-X) that was used corresponded to the entire extracellular domain of the Mpl receptor minus Trp at position 483 (*See* Vigon, et al, 8: 2607-2615 (1993)). In order to optimize binding of Mpl ligand to the Mpl-X receptor affinity column, the WGA elution pool was concentrated using a membrane ultrafilter (10,000 molecular weight cut off, YM-10, Amicon) and NaCl adjusted to 0.2 M by subsequent dilution. The concentrated WGA pool was applied to a 20 ml m-Mpl-X (murine Mpl soluble receptor)/CNBr activated Sepharose column (2.6 x 4.2 cm, 1.5 mg m-Mpl-X per ml of resin) at a flow rate 0.9 ml/min. The column was washed with 40 ml of PBS/azide at 1.5 ml/min, followed by a high salt wash (405 ml) with 10 mM Tris-HCl, 1 M NaCl, 1 mM CHAPS, pH 8.0. The

column was then eluted with 20 mM CAPS, 1 M NaCl, 5 mM CHAPS, pH 10.5. Appropriate fractions were collected. Tris was added to each fraction to neutralize the pH.

Both the SDS-PAGE and the absorbance at 280nm of the elution profile of an Mpl-X receptor affinity column reveal an early protein peak in fractions 1-4, while the majority of the Mpl ligand activity eluted after fraction 5.

D. Mono-Q Anion exchange chromatography.

The highest purity fractions from several Mpl-X receptor affinity columns were combined, concentrated, and diafiltered against 20 mM Tris-HCl, 5 mM CHAPS, pH 8.7 to a final volume of 58.5 ml. The protein concentration of the pool was estimated by absorbance at 280nm to be 0.12 mg/ml (approximately 7 mg total protein). The pool was loaded at 0.5 ml/min onto a Mono Q HR 5/5 column (Pharmacia) equilibrated in 20 mM Tris-HCl, 5 mM CHAPS, pH 8.7. The column was eluted with a linear gradient to 0.36 M NaCl in the same buffer over 27 minutes. The column was then washed with a 6 minute gradient to 0.54 M NaCl, and finally with a step wash at 0.9 M NaCl. One ml fractions were collected.

The elution profile of the Mono Q column shows that no Mpl ligand, and negligible protein, could be detected in the flow-through and wash fractions. Much of the Mpl ligand activity elutes in fractions 5-7, during the initial stages of the NaCl gradient. A "shoulder" of activity is observed in fractions 8-10, followed by a second major peak comprising fractions 11-15.

A distinct 25 kd band is observed by SDS-PAGE (nonreducing) in the active fractions. The intensity of the band directly corresponds with the Mpl ligand activity in the fractions. The band was absent in fractions 3 and 4 (no activity). It was prominent in

fractions 5 and 6 (1A6.1 activity peak) and a similar, intensely stained band, was present in fractions 11-14 (1A6.1 activity peak). The band is faint in the pool of fractions 15 and 16, corresponding with the significantly lower activity in fraction 16.

E. Gel Elution Experiments.

Gel elution experiments were performed using aliquots of Mono Q fractions 5 and 6 or Mono Q fractions 13 and 14. For these experiments, pools of fractions 5 and 6 (6 µl each) or 13 and 14 (7.5 µl each) were made, mixed with SDS-PAGE sample buffer (nonreducing), and applied to 12% SDS gels. Upon completion of electrophoresis, lanes of interest were sliced (1 mm) and the slices were diced into small pieces with razor blades. The pieces were transferred to 1.5 ml microfuge tubes containing 0.5 ml PBS/5mM CHAPS and gently agitated overnight at 4°C. The next day the tubes were spun briefly, an aliquot was removed, and the sample was diafiltered against Iscove's medium supplemented with BSA as a carrier protein. The diafiltered samples were submitted for assay.

The results reveal that two peaks of Mpl ligand activity can be observed. One peak corresponds to the 25 kd region of the gel, while a second peak of Mpl ligand activity is observed in the 31 kd region.

F. Superdex 200 Gel Filtration.

Fractions 13-16 from the Mono Q anion exchange column, as well as two equivalent fractions from a second Mono Q fractionation, were combined and concentrated using a membrane ultrafilter (Centricon-10, Amicon). SDS was added to a final concentration of 0.1%, and the sample was injected onto a Superdex 200 HR 10/30 (Pharmacia) column. The column was equilibrated in 50 mM Tris-HCl, 0.1% SDS, pH 7.5 at a flow rate of

0.3 ml/min, and was operated at room temperature. One minute fractions were collected. The results were that most of the protein in the sample elutes in fractions 32-40, while the Mpl ligand activity is detected in fractions 42-46. Analysis of fractions SDS-PAGE showed a distinct 25 kd band in the active fractions.

G. C4 Reversed Phase HPLC.

Superdex 200 fractions 43-46 combined or fraction 42 alone were concentrated using a membrane ultrafilter (Microcon-10, Amicon). The concentrated pools were separately applied to a 1 x 100 mm C4 reversed phase microbore column (SynChropak RP-4). The column was equilibrated in 0.04% TFA in water (A Buffer); B Buffer was 0.035% TFA in 80% acetonitrile. After injection of the sample, a linear gradient to 45% B over 4 min was performed, followed by a linear gradient to 75% B over 40 min. Flow rate was 75 µl/min. The results of purification of fraction 42 are presented in FIG. 5. Distinct Mpl ligand activity peaks were observed in fractions 21-23. These fractions were analyzed on a 14% polyacrylamide gel under nonreducing and reducing conditions. Fraction 21 was composed of a single 31 kd band; fraction 23 was composed of a single, broad 25 kd band; and fraction 22 contained bands in both the 25 kd and 31 kd regions. No other significant bands were visible. Note that earlier gel elution experiments had ascribed Mpl ligand activity to both of these regions. A single, minor high molecular weight band was observed in all fractions of the nonreducing gel, but could not be seen in the reducing gel.

H. <u>N-terminal Sequence Analysis of 25 kd and 31 kd Mpl ligands</u>.

N-terminal sequence analysis was carried out on C4 RP-HPLC fractions containing activity. The sequences determined for these proteins are reported above. In addition to the major sequence corresponding to the 25 kd band (at least 90% of the total of the applied sample), sequencing detected two minor sequences (which were associated with the minor contaminating band described in part G above). Comparisons with known sequences revealed that the minor sequences were canine Ig heavy chain and kappa chain. If desired, these minor impurities could be further reduced in quantity by application of another purification step, such as preferably another gel filtration step.

I. <u>Comparison of Mpl ligand activities in the C4 purified fractions</u>

FIG. 6 shows data demonstrating that the activities present in fractions 22 and 23 from the C4 RP-HPLC chromatography step are substantially equivalent. Fraction 22 contained a mixture of the 25 and 31 kd bands, whereas fraction 23 contained only the 25 kd band. Aliquots of each fraction were diluted 1:45000. The diluted fractions stimulated 1A6.1 cell growth substantially equally, (fraction 22, 5400 cells per well; fraction 23, 6000 cells per well). The diluted fractions were incubated with increasing concentrations of Mpl-X. The fractions were equally sensitive to inhibition by Mpl-X, both being completely blocked with 7-1.4 ug/ml. This indicates that the active protein(s) in each fraction are Mpl ligand species with equivalent biological activity.

EXAMPLE 8

<u>Comparison of Mpl ligand to other factors on megakaryocyte development</u>

A number of recombinant factors or organic compounds such as phorbol myristic acetate (PMA) have been reported to impact megakaryocyte growth or development. Accordingly, the effects of these factors on CD34-selected peripheral blood cells were investigated. Human recombinant interleukin 3 (IL-3, 1-2 ng/ml), stem cell factor (SCF, 50 ng/ml), interleukin 6 (IL-6, 25 ng/ml), erythropoietin (EPO, 1 Unit/ml), leukemia inhibitory factor (LIF, 10 ng/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF, 25 ng/ml, Amgen, Inc.); interleukin 11 (IL-11, 25 ng/ml, R+D Systems, Minneapolis, MN); phorbol myristic acetate (PMA, 10^{-10} M, Sigma) were added to cultures as indicated. Mpl ligand was used at 275 units per ml, APK9 was used at 5% (equivalent to 220 units/ml). Factors tested in combination were at the same concentration as when tested individually. After 8 days in culture, the cells were fixed directly in the wells and stained for megakaryocytes (n=6 wells per condition) or counted for total cell number (n=3 wells per condition). Data are presented as mean +/- SEM.

FIG. 7 shows that APK9 and Mpl ligand resulted in the greatest number of megakaryocytes per well. IL-3 also resulted in megakaryocyte development, especially in combination with SCF. IL-6, IL-11, or EPO had little effect on megakaryocyte numbers either alone or in combination with IL-3. PMA, LIF and GM-CSF had little effect. In FIG. 8 are data from the same experiment showing the total number of cells found per well ("cellularity"). APK9 and Mpl ligand had little effect on cellularity while IL-3 and SCF had modest effects.

SCF and IL-3 in combination had the greatest effects. The data shown in FIGS. 7 and 8 were used to calculate percentages of megakaryocytes per well, as shown in FIG. 9. Clearly, the factor which results in the greatest percentage of megakaryocytes per culture well is Mpl ligand, the active ingredient in APK9. This is indicative of the specificity of Mpl ligand towards megakaryocytes.

EXAMPLE 9

The megakaryocyte promoting activity of Mpl ligand is not dependent on human IL-3

Mpl ligand stimulates the development of human megakaryocytes when used as a supplement to the culture medium described in Example 2. Although IL-3 is not an ingredient of the medium, it could be present in undetectably low levels in the normal human plasma present in the medium. However, even if present, IL-3 is not involved in Mpl ligand-induced megakaryopoiesis. This is shown in FIG. 10. IL-3 at 2 ng/ml contains an activity in the human meg assay of 14,900 meg units/ml. This activity is 97% inhibited with anti-IL-3 (3.3 ug/ml; Genzyme, Cambridge, MA). MPL ligand at 8203 meg units/ml was not inhibited with anti-IL-3.

EXAMPLE 10

Analysis of porcine Mpl ligand

I. Summary

Proteins from irradiated pig plasma with Mpl ligand activity were characterized with WGA affinity chromatography, Mpl receptor affinity chromatography,

ion exchange chromatography and C4 reverse phase HPLC. The activity was also characterized by elution from slices from SDS-polyacrylamide gels.

Chromatography	Comments
WGA affinity column	4.4×10^6 units applied. 3.4×10^6 units recovered
Mpl receptor column	2.7×10^6 units applied 2.4×10^6 units recovered
Mono S ion exchange pH 6.0	2.4×10^6 units applied 4.4×10^6 units recovered
C4 reverse phase HPLC	Activity recovered fractions 23-25
Gel elution Experiments	Two activities clearly distinguished, one at approximately 18 kd, the other at approximately 28 kd.

EXAMPLE 11

Cloning of the Human Mpl-ligand, Human MGDF

Two approaches are outlined in the following:

I. First Exemplary Cloning Approach

A. Generation of human MDGF probe

A number of degenerate PCR primers were designed based on the amino terminus sequence of the canine protein. Different primer pairs were used to amplify the MGDF gene from the human genomic DNA. After 40 cycles of amplification, using the sense primers 5' GCN CCN CCN GCN TGY GA 3'(SEQ ID NO: 4), encoding the first five amino acids of the canine protein (SEQ ID NO: 1) and the antisense primer: 5' GCA RTG YAA CAC RTG NGA RTC 3'(SEQ ID NO: 5), encoding amino acids 16 to 21 of SEQ ID NO: 1, the PCR product was run on a 2.0% agarose gel in TBE buffer.

The 63 bp band was cut out from the agarose gel and reamplified using the same primer set. The PCR product was cloned in the PCR II vector (Invitrogen, San Diego). A number of colonies were screened by DNA sequencing. The plasmid DNA encoding a peptide similar to the canine MGDF protein was used as the source to generate a radioactive probe to screen the cDNA libraries. The amino acid sequence encoded by the gene fragment is as follows:

Ala-Pro-Pro-Ala-Cys-Asp-Leu-Arg-Val-Leu-Ser-Lys-Leu-Leu-Arg-Asp-Ser-His-Val-Leu-His (SEQ ID NO: 6)

The agarose band containing the human MGDF was used to generate the probe by hot PCR. A typical PCR

reaction of 100 µl contained the following ingredients:

template DNA	2-3 µl
5' primer (SEQ ID NO:4)	1 µl, 20 pmoles
3' primer (SEQ ID NO:5)	1 µl, 20 pmoles
10 X buffer	10 µl
dATP (0.1 mM)	2 µl
dTTP (10 mM)	2 µl
dGTP (10 mM)	2 µl
dCTP (0.1mM)	2 µl
dCTP, p^{32} (10 uC/ul)	5 µl
dATP, p^{32} (10 uC/ul)	5 µl
Taq DNA polymerase	0.5 µl, 2.5 units
water	77 µl
total volume	100 µl

The amplification conditions were as follows:

initial heating	94°C, 2 min
anneal	53°C, 30 sec
extension	72°C, 30 sec
denaturation	94°C, 30 sec.

40 cycles of amplification was carried out in a Perkin Elmer GeneAmp System 9600.

The product was purified by passing through a push column (Stratagene, San Diego). One 1 µl of the probe was counted in a scintillation counter. Probes containing 1 to 3 million counts per ml were added to the hybridization mix.

B. Construction of fetal liver library

Human fetal liver polyA+ RNA was purchased from Clontech laboratories. About 4 µg of RNA was used for cDNA synthesis, in which priming was carried out using a random hexamer, 5' GTA CGC GTT CTA GAN NNN NNT 3', (SEQ ID NO: 7) attached to an oligo containing an Xba I site.

The Gibco-BRL protocol was used to generate the double stranded cDNA. The Eco R I-Bst X I adaptor (Invitrogen, San Diego) was ligated to the double stranded cDNA, followed by digestion with the restriction enzyme, Xba I. Size selection of the cDNA was carried out on a S500 Sephacryl column (Life Technologies, Inc.). cDNAs larger than 400 bp were ligated to the mammalian expression vector v19.8 (Martin, F., *Cell* **63**: 203-211 (1990)) which was already digested with Eco RI and Xba I. Competent *E. coli* DH10 cells were transformed and the resulting cDNA library was split into 7 pools of 100,000 cDNA each.

C. Screening the lambda library

A human fetal kidney library in lambda gt11 was bought from Clontech with a titer of 650 million pfu/ml. About 2 million plaques were screened with a probe generated by PCR (see above). Hybridization was done in 6 X SSC, 5 X Denhardt, 0.1% SDS, 100 µg/ml single strand salmon sperm DNA for 15 hours at 56°C.

Multiple rounds of screening were carried out. DNA was amplified from single plaques and hybridized with the internal primer 5' AGT TTA CTG AGG ACT CGG AGG 3'(SEQ ID NO: 8) encoding amino acids 7 to 13 in SEQ ID NO: 6 to identify the true positives.

D. 3 prime Rapid Amplification of cDNA Ends (RACE)

Polyadenylated RNA from human fetal kidney and fetal liver were bought from Clontech. One microgram RNA was reverse transcribed using the oligo 5' TTC GGC CGG ATA GGC CTT TTT TTT TTT TTT 3'(SEQ ID NO: 9) as the primer.

The Gibco-BRL cDNA synthesis kit (Life Technologies Inc., Cat. # 18267-013) was used to generate the first strand cDNA. The final volume was 30 µl. The reaction was stopped by adding 500 mM EDTA to a final concentration of 10 mM and kept at -20°C.

For initial PCR, 0.5 µl of cDNA was used as the template per reaction. The primer SEQ ID NO: 9 and the competitor oligo 5' TTC GGC CGG ATA GGC CTT TTT TTT TTT TT-P 3'(SEQ ID NO: 10) were used as the antisense primers, while the oligonucleotide 5' TGC GAC CTC CGA GTC CTC AG 3'(SEQ ID NO: 11) encoding amino acids 5 to 11 of SEQ ID NO: 6, was used as the sense primer. Forty cycles of amplification were carried out using the following protocol: 94°C, 30 sec; 65°C, 30 sec; 72°C, 30 sec, after an initial 2 min incubation at 94°C. A Perkin Elmer GeneAmp System 9600 was used for amplification.

Nesting was carried out using the sense primer 5' GAG TCC TCA GTA AAC TGC TTC GT 3'(SEQ ID NO: 12) encoding amino acids 8 to 14 of SEQ ID NO: 6, while SEQ ID NO: 9 and SEQ ID NO: 10 served as the antisense primers. Forty cycles of amplification were carried out with annealing at 65°C. The PCR products were run on a 0.8% agarose gel and then photographed under UV light. Bands around 0.8 to 1.2 kb were visible.

The PCR products were then cloned in the vector PCR II (Invitrogen). Individual colonies were picked and plasmids were isolated using the Qiagen kits

cat # 12143 and 12145. Double stranded dye primed sequencing was done using the vector primers. The sequences were analyzed by various types of GCG software.

E. 5' and 3' primer extension

In order to isolate the sequence of the full length MGDF gene, 3' and 5' primer extensions were carried out using different pools of fetal liver library as the template. For the amplification of the 5 primer of the cDNA, about 20 ng of cDNA from each pool was used as the template. A MGDF specific antisense primer 5' GGA GTC ACG AAG CAG TTT AC 3'(SEQ ID NO: 13) encoding amino acids 12 to 17 of SEQ ID NO: 6 and the 5' vector v19.8 sense primer 5' CCT TTA CTT CTA GGC CTG 3'(SEQ ID NO: 14) were used. Amplification was carried out for 30 cycles with annealing at 53°C. Nesting was done for 30 cycles with the antisense primers 5' GAG GTC ACA AGC AGG AGG A 3'(SEQ ID NO: 15) encoding amino acids 1 to 6 of SEQ ID NO: 6 and the vector primer SEQ ID NO: 14.

For the primer extension of the 3' ends of the MGDF cDNAs, the antisense vector primer 5' GGC ATA GTC CGG GAC GTC G 3'(SEQ ID NO: 16) and the MGDF specific primer 5' TCC TCC TGC TTG TGA CCT C 3'(SEQ ID NO: 17) encoding amino acids 1 to 6 of SEQ ID NO: 6, were used. Amplification was carried out for 30 cycles with annealing at 58°C.

Nesting amplification for 30 cycles was done using the MGDF primer SEQ ID NO: 12 and the vector primer SEQ ID NO: 16. Specific bands appeared in pool numbers 1, 7 and 8, which were cloned in the PCR II vector. Purified plasmid DNA from single colonies was purified and sequenced.

F. Isolation of full length clones of human MGDF

Many of the initial clones lacked part of the amino terminus of MGDF, since part of the MGDF sequence was used for priming and nesting. Primer 5' CCA GGA AGG ATT CAG GGG A 3'(SEQ ID NO: 18), whose sequence was obtained from the 5 primer extension experiments as described above was used as the sense primer. The vector primer SEQ ID NO: 16 served as the antisense primer. 35 cycles of amplification was carried out with annealing at 58°C. MGDF specific primer 5' CAA CAA GTC GAC CGC CAG CCA GAC ACC CCG 3'(SEQ ID NO: 19) with a Sal I site and the vector primer (SEQ ID NO: 15) were used for nesting for 35 cycles. The PCR product was cloned in PCR II vector and sequenced.

II. Second Exemplary Cloning Approach

A. Cloning Of Canine MGDF N-Terminus cDNA

Degenerate oligonucleotide primers were designed based on the canine MGDF N-terminus amino acid sequence described in the previous section and used as primers in polymerase chain reactions (PCRs) to amplify MGDF-encoding cDNA sequences. Total RNA was prepared from canine kidney samples by the guanidinium isothiocyanate method of Chomzynski and Sacchi (*Biochem.* **162**: 156-159 (1987)). First strand cDNA was prepared with a random primer-adapter 5' GGC CGG ATA GGC CAC TCN NNN NNT 3' (SEQ ID NO: 20) using MoMULV reverse transciptase and used as template in subsequent PCRs.

PCR was performed on 0.5 microliters, about 50 ng, of the cDNA, using Primer A 5' GCN CCN CCN GCN TGY GA 3' (SEQ ID NO: 4), a sense strand primer encoding amino acids 1-6 of SEQ ID NO: 1, and either primer B 5' GCA RTG NAG NAC RTG NGA RTC 3' (SEQ ID NO: 5) or primer

C 5'GCA RTG YAA NAC RTG NGA RTC 3' (SEQ ID NO: 21), which are antisense strand primers encoding amino acids 16-21 of SEQ ID NO: 1 with three extra nucleotides at the 5' termini to increase annealing stability. PCR with Taq polymerase was performed for 35 to 45 cycles, until product bands were apparent on agarose gel electrophoretic analysis. For the first two cycles of PCR, the reannealing step was performed at 37°C for 2 minutes; for the remainder of the cycles reannealing was at 50°C for 1 minute. Multiple product bands were observed in each reaction. Portions of the gel containing bands of approximately the expected size (66 bp) were collected with the tip of a Pasteur pipette and re-amplified with the same primer pair. The DNA products were cloned into vector PCR II (Invitrogen) according to the manufacturer's instructions. Three clones were sequenced and were found to encode, in one reading frame, the expected canine MGDF sequence, residues 1-21. In this way unique canine MGDF cDNA sequence was obtained spanning the region from the third nucleotide of codon 6 through the third nucleotide of codon 15. One of these clones served as the template for preparation of a labeled canine MGDF cDNA probe.

B. Construction of cDNA library from human fetal liver

RNA has been isolated from human fetal Liver (International Institute for the Advancement of Medicine, Exton, PA) by lysis of tissue in 5.5 M guanidinium thiocyanate and purification via CsTFA (Pharmacia) centrifugation. Polyadenylated RNA was selected using oligo $(dT)_{25}$ dynabeads (Dynal, according to manufacturer's instruction). Double stranded cDNA was produced from this RNA using Superscript plasmid system for cDNA synthesis (Life Technologies, Inc.) except a different linker adapter: 5' TTG GTG TGC ACT

TGT G 3' (SEQ ID NO: 22) and 5' CAC AAG TGC ACA CCA ACC CC 3'(SEQ ID NO: 23), was used. After size selection this cDNA was directionally inserted into the Bst XI and Not I sites of the mammalian expression vector pBCB (pBCB is derived from the plasmid Rc/CMV, Invitrogen, comprising the puc19 backbone, CMV promoter and BGH polyadenylation site). The ligated DNA was electroporated into electro competent bacterial strain 10B (Life Technologies, Inc.).

C. Screening of human fetal liver cDNA library for MGDF

Filter replicas of the human fetal liver library were hybridized to radioactively labeled canine MGDF N-terminus cDNA PCR product (5x SSPE, 2x Denhardt's, 0.05% Na pyrophosphate, 0.5% SDS, 100 µg/ml yeast tRNA lysate and 100 µg/ml denatured salmon sperm DNA) at 64°C for 18 h. Filters were washed at 64°C in 5x SSPE, 0.5% SDS and exposed over night. Two different clones hybridizing to this probe were isolated and analyzed.

D. Expression of human MGDF cDNA clones

Purified DNA from MGDF cDNA clones was transfected into 293 EBNA cells (Invitrogen). 1.5 µg of DNA was mixed with 7.5 ul Lipofectamine (Life Technologies, Inc.) in 100 ul of serum free DMEM. After a 20 minute incubation at room temperature the DNA-Lipofectamine mixture was added to 5×10^5 cells/well (24 well square Greiner plate) in 400 ul DMEM, 1% serum (Fetal Clone II) and incubated for 6 hours at 37°C. 500 ul DMEM, 20% serum (Fetal Clone II) was added to the cells. 16 hours later the media was aspirated and 500 ul DMEM, 1% serum (Fetal Clone II) was added. 72 hours later the conditioned media were collected and centrifuged through a 0.22 micron spin-filter. The conditioned

media were assayed for MGDF biological activity.

III. Description and Activity of Human MGDF Clones

Based on the above-described cloning strategies, the human cDNA clones shown in FIG. 11 (MGDF-1 and MGDF-2; SEQ ID NOS: 24, 25; and 26, 27) and FIG. 12 (MGDF-3; SEQ ID NOS: 28, 29) were obtained. Each of these sequences in the Figures contains a putative signal sequence of amino acids 1-21, so the mature proteins start at amino acid 22 in each case.

The results of activity assays using the cell-based assay described in Example 4A above with MGDFs 1-3 are presented in Tables 3 and 4 below. In Table 3, conditioned media from 293 EBNA cells transfected with each construct was collected after 2 days of culture then tested on 1A6.1 cells (32D/mur-MPL+) +/- 10 ug/ml mur-MPL-X. In Table 4, conditioned media from 293 EBNA cells transfected with each construct was collected after 4 days of culture then tested on both 32D/mur-MPL+ cells (Example 3A) and 32D/hu-MPL+ cells (Example 3B). As can be seen, human MGDF-1 and MGDF-2, but not MGDF-3, were found to be active on cell lines expressing both the murine and human forms of Mpl. The cell line expressing the human MPL receptor is more responsive to human MGDF-1 and MGDF-2 than is the cell line expressing the murine Mpl receptor.

Table 3

Clone	U/ml (- mur-MPL-X)	U/ml (+ mur-MPLX)
Media	0	0
PBCO (control plasmid)	0	0
MGDF-1	12,800	800
MGDF-1 (repeat)	12,800	566
MGDF-2	4525	400
MGDF-2 (repeat)	12800	1131
MGDF-3	0	0
MGDF-3 (repeat)	0	0
APK9 control	4400+/-400	0

Table 4

Clone	U/ml 32D/mur-MPL+	U/ml 32D/hu-MPL+
MGDF-1	1600	25,600
MGDF-2	6400	50,000
MGDF-2 (repeat)	6400	50,000-100,000

The following Table 5 shows that the activities of human MGDF-1 and MGDF-2 on 32D/hu-MPL+ cells (Example 3B) are substantially completely inhibited by soluble human mpl receptor (hu-MPL-X). Hu-MPL-X was present as conditioned media collected from CHO cells producing the protein. The CHO hu-MPL-X conditioned media was concentrated 120-times then added to the cultures at 6.6%. Conditioned media from control

CHO cultures had no effect on the assay. The assay was carried out as described in Example 4B except that the viable cells were assessed after 3 days.

Table 5

Clone	U/ml (-Hu-MPL-X)	U/ml (+Hu-MPL-X)
MGDF-1	530	0
MGDF-2	270	0

Human Megakaryocyte Assay

MGDF-1 and MGDF-2 but not MGDF-3 induced the formation of megakaryocytes from peripheral blood CD34-selected cells. The experiment described in Table 6 was performed essentially as described in Example 2 except that peripheral blood cells were CD34-selected without elutriation and the culture was harvested after 7 days. Conditioned media from each 293 EBNA MGDF construct was used at 20% final volume +/- 30 ug/ml mur-MPL-X. APK9 control was used at 6% final volume.

Table 6

Clone	Megakaryocytes per Well (-mur-MPL-X)	Megakaryocytes per Well (+mur-MPL-X)
vector control	0	0
APK9 control	100 +/- 3	0
MGDF-1	142 +/- 48	17 +/- 2
MGDF-2	100 +/- 3	6 +/- 2
MGDF-2 repeat	86 +/- 10	0
MGDF-3	2 +/- 2	0

EXAMPLE 12

The following example describes the synthesis of 12 different pegylated MGDF molecules, PEG 9 - PEG 12 and PEG 14 - PEG 21. In each case, the MGDF molecule that was pegylated was *E. coli* derived MGDF-11 (amino acids 22-184, numbering from the beginning of the signal peptide or amino acids 1-163, numbering from the beginning of the mature protein). Details regarding all of these pegylated species are summarized in Tables 7-10 below.

12.1 Preparation of poly-MePEG-MGDF conjugates by MGDF acylation with activated MePEG derivatives

Preparation of poly-MePEG(20kDa)-MGDF conjugate (PEG 11).

A cooled (4°C) solution of MGDF (2.5 mg/ml) in 0.1 M BICINE buffer, pH 8, was added to a 10-fold molar

excess of solid MePEG succinimidyl propionate (MW 20 kDa) (Shearwater Polymers, Inc.). The polymer was dissolved by gentle stirring and the reaction further conducted at room temperature.

The extent of the protein modification during the course of the reaction was monitored by size exclusion (SEC) HPLC using Superdex 200 HR 10/30 column (Pharmacia Biotech) eluted with 0.1 M sodium phosphate buffer pH 6.9 at 0.7 ml/min.

SEC HPLC analysis of the reaction mixture at the 30 minute time point indicated that no free protein was left in the reaction mixture. At this point the protein concentration in the reaction mixture was reduced to 1 mg/ml by addition of sterile water and the pH of the mixture adjusted to 4 with several drops of 0.5M acetic acid.

MePEG-MGDF conjugate was separated from the excess of MePEG and other reaction by-products by ion-exchange chromatography using SP Sepharose HP (Pharmacia Biotech) ion exchange resin.

The reaction mixture was loaded (2.5 mg/ml of resin) onto the column and the unreacted MePEG was eluted with 3 column volumes of the starting buffer A (20 mM sodium phosphate, pH 7.2, 15% glycerol). After that, the MePEG-MGDF conjugate was eluted using a linear gradient from 0% to 30% in 10 column volumes of the end buffer B (1M NaCl in buffer A). The eluent was monitored at 280 nm. Fractions containing poly-MePEG-MGDF conjugate were pooled, concentrated and sterile filtered.

The purified poly-MePEG-MGDF conjugate was analyzed by HPLC SEC using TSK-GEL G4000SWXL and G2000SWXL gel filtration columns coupled in series. Proteins were detected by UV absorbance at 280 nm. BIO-RAD gel filtration standards served as globular protein

molecular weight markers.

As can be seen in FIG. 17A, HPLC SEC reveals two major components in the preparation (in about a 2 to 1 ratio) elution positions of which correspond to those of 370.9 kDa and 155.0 kDa globular proteins respectively. See also Table 8 below.

Conjugates PEG 9, PEG 10 and PEG 12 prepared by MGDF acylation with succinimidyl esters of MW=6-50 kDa MePEGs were conducted similarly. The major reaction parameters used in these preparations are summarized in Table 7.

Results of HPLC SEC analyses of these conjugates are shown in Table 8.

12.2. <u>Preparation of poly-MePEG-MGDF conjugates by MGDF reductive alkylation with MePEG aldehydes</u>.

<u>Preparation of poly-MePEG(20kDa)-MGDF conjugate (PEG 20)</u>.

To a cooled (4°C), stirred solution of MGDF (2 ml, 2.5 mg/ml) in 100 mM sodium phosphate, pH 5, containing 20 mM $NaCNBH_3$ was added a 10-fold molar excess of monomethoxy-polyethylene glycol aldehyde (MePEG) (average molecular weight 20 kDa) and the stirring of the reaction mixture was continued at the same temperature.

The extent of the protein modification during the course of the reaction was monitored by SEC HPLC using Superdex 200 HR 10/30 column (Pharmacia Biotech) eluted with 0.1 M sodium phosphate buffer pH 6.9 at 0.7 ml/min.

After 16 hours the SEC HPLC analysis indicated that more than 90% of the initial amount of the protein has been modified. At this time the protein concentration in the reaction mixture was brought to 1

mg/ml by dilution of the reaction mixture with sterile water and the pH adjusted to 4 (0.5M acetic acid).

MePEG-MGDF conjugate was separated from the excess of MePEG and other reaction by-products by ion-exchange chromatography using SP Sepharose HP (Pharmacia Biotech) ion exchange resin.

The reaction mixture was loaded (2.5 mg/ml of resin) onto the column and the unreacted MePEG was eluted with 3 column volumes of the starting buffer A (20 mM sodium phosphate, pH 7.2, 15% glycerol). After that, the MePEG-MGDF conjugate was eluted using a linear gradient from 0% to 30% in 10 column volumes of the end buffer B (1M NaCl in buffer A). The eluent was monitored at 280 nm. Fractions containing poly-MePEG-MGDF conjugate were pooled, concentrated and sterile filtered.

The purified poly-MePEG-MGDF conjugate was analyzed by HPLC SEC using TSK-GEL G4000SWXL and G2000SWXL gel filtration columns coupled in series. Proteins were detected by UV absorbance at 280 nm. BIO-RAD gel filtration standards served as globular protein molecular weight markers.

As can be seen in FIG. 17B, HPLC SEC reveals two major components (constituting 52% and 47% of the total amount) in the preparation, elution positions of which correspond to those of 359.4 kDa and 159.3 kDa globular proteins respectively. See also Table 8.

Conjugates PEG 18, PEG 19 and PEG 21 prepared by MGDF reductive alkylation with MePEG aldehydes of MW=6-25 kDa were conducted similarly. The major reaction parameters used in these preparations are summarized in Table 7.

Results of HPLC SEC analyses of these conjugates are shown in Table 8.

12.3. <u>Preparation of monomethoxy-polyethylene glycol-MGDF conjugates with the site of attachment at the N-terminal α-amino residue</u>.

<u>Preparation of mono-MePEG (20kDa)-MGDF conjugate (PEG 16)</u>.

To a cooled (4° C), stirred solution of MGDF (2 ml, 2.5 mg/ml) in 100 mM sodium phosphate, pH 5, containing 20 mM $NaCNBH_3$ was added a 5-fold molar excess of methoxypolyethylene glycol aldehyde (MePEG) (average molecular weight 20 kDa) and the stirring of the reaction mixture was continued at the same temperature.

The extent of the protein modification during the course of the reaction was monitored by SEC HPLC using Superdex 200 HR 10/30 column (Pharmacia Biotech) eluted with 0.1 M sodium phosphate buffer pH 6.9 at 0.7 ml/min.

After 16 hours the SEC HPLC analysis indicated that about 90% of the initial amount of the protein has been modified. At this time the protein concentration in the reaction mixture was reduced to 1 mg/ml by dilution with sterile water and the pH of the reaction mixture adjusted to 4 (0.5 M acetic acid).

The mono-MePEG (20kDa)-MGDF conjugate was separated from the excess of MePEG and other reaction by-products by ion-exchange chromatography using SP Sepharose HP (Pharmacia Biotech) ion exchange resin.

The reaction mixture was loaded (2.5 mg/ml of resin) onto the column and the unreacted MePEG was eluted with 3 column volumes of the starting buffer A (20 mM sodium phosphate, pH 7.2, 15% glycerol). After that, the MePEG-MGDF conjugate was eluted using a linear gradient from 0% to 25% of the end buffer B (1M NaCl in buffer A) in 20 column volumes. The eluent was monitored at 280 nm. Fractions containing poly-MePEG-

MGDF conjugate were pooled, concentrated and sterile filtered.

The homogeneity of the mono-MePEG-MGDF conjugates was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis using 4-20% precast gradient gels (NOVEX). One major band corresponding to the position of a 46.9 kDa protein was revealed.

The purified poly-MePEG-MGDF conjugate was analyzed by HPLC SEC using TSK-GEL G4000SWXL and G2000SWXL gel filtration columns coupled in series. Proteins were detected by UV absorbance at 280 nm. The BIO-RAD gel filtration standards served as globular protein molecular weight markers.

As can be seen in FIG. 17C, SEC HPLC reveals one major component in the preparation, elution positions of which corresponds to that of 181.1 kDa globular protein. See also Table 9.

Mono-MePEG-MGDF conjugates PEG 14, PEG 15 and PEG 17 prepared by MGDF reductive alkylation with MePEG aldehydes of MW=6-25 kDa were conducted similarly. The major reaction parameters used in these preparations are summarized in Table 7.

Results of HPLC SEC analyses of these conjugates are shown in Table 9.

Table 7

Summary of MGDF modification reaction parameters

Code	Reactive MePEG		Reaction conditions				
	Type	MW	MGDF conc. mg/ml	pH	Temperature, °C	Time, h	Molar Ratio MePEG/MGDF
PEG 9	NHS ester	6kDa	2.5	8	r.t.	0.5	15
PEG 10	NHS ester	6kDa	2.5	8	r.t.	0.5	10
PEG 11	NHS ester	20kDa	2.5	8	r.t.	0.5	10
PEG 12	NHS ester	50kDa	2.5	8	r.t.	0.5	5
PEG 14	ALDEHYDE	6 kDa	2.5	5	4°C	16	5
PEG 15	ALDEHYDE	12kDa	2.5	5	4°C	16	5
PEG 16	ALDEHYDE	20kDa	2.5	5	4°C	16	5
PEG 17	ALDEHYDE	25kDa	2.5	5	4°C	16	10
PEG 18	ALDEHYDE	6kDa	5	5	4°C	16	10
PEG 19	ALDEHYDE	12kDa	5	5	4°C	16	10
PEG 20	ALDEHYDE	20kDa	5	5	4°C	16	10
PEG 21	ALDEHYDE	25kDa	5	5	4°C	16	10

Table 8

Summary of poly-MePEG-MGDF characteristics by SEC HPLC

Code	Reactive MePEG		Apparent MW by SEC, kDa	Component amount, %
PEG 9	NHS ester	6kDa	87.9	75
			52.7	25 (shoulder)
PEG 10	NHS ester	6kDa	69.2	14 (shoulder)
			42.9	86
PEG 11	NHS ester	20kDa	370.9	68
			155.0	32
PEG 12	NHS ester	50kDa	865.6	53
			368.0	47
PEG 18	ALDEHYDE	6kDa	84.6	60
			41.5	40
PEG 19	ALDEHYDE	12kDa	218.4	59
			106.7	41
PEG 20	ALDEHYDE	20kDa	359.4	52
			159.3	47
PEG 21	ALDEHYDE	25kDa	450.5	54
			218.4	46

Table 9

Apparent molecular weights of mono-MePEG-MGDF Conjugates

Code	Reactive MePEG Type	Reactive MePEG MW	Apparent MW by SEC, kDa	Apparent MW by SDS PAGE, kDa
PEG 14	ALDEHYDE	6 kDa	44.5	27.7
PEG 15	ALDEHYDE	12kDa	104.7	38.3
PEG 16	ALDEHYDE	20kDa	181.1	46.9
PEG 17	ALDEHYDE	25kDa	226.4	55.5

12.4. <u>Preparation of DiMePEG (12kDa)-MGDF conjugates by reductive alkylation of MGDF with methoxy poly(ethylene glycol) aldehyde (PEG 22)</u>.

The following procedure results in a purified molecule referred to herein as PEG 22.

A 5-fold excess of methoxy polyethylene glycol aldehyde (MePEG; i.e., $OHC-(CH_2)_2O-(CH_2-CH_2O)_n-CH_3$; where n = a repeat such that the molecular weight is ca. 12 kDa) (Shearwater Polymers), was added to a 2.5 mg/mL solution of MGDF (*E. coli* derived, 1 - 163) in 100 mM sodium acetate, pH 5.0 held at 5 degrees Celsius. After mixing for 10 minutes, sufficient sodium cyanoborohydride (Aldrich) was added to achieve a 20 mM concentration in the reaction mixture.

This mixture was stirred for 16 hours at

approximately 5 degrees C. At the end of this time, sufficient purified water, USP was added to bring the concentration of MGDF to 1 mg/mL. This was filtered through a 0.2 micron vacuum filter. 90 mg of reaction product were prepared in this manner. Small amounts of 1.0 M monobasic phosphate and 1 N sodium hydroxide solutions were added to the reaction product mixture to achieve a 10 mM phosphate, pH 6.8 solution.

The conjugate was purified on a cation exchange column. A 40mL SP-Sepharose High Performance column was prepared with a bed height of 7.5 cm. The column was equilibrated with equilibration buffer (10 mM phosphate, pH 6.8, with 15% glycerol). The column was loaded at 2.2 mg/mL resin at 0.15 column volumes (CV) per minute. This was followed by a wash with the equilibration buffer until baseline was achieved. The column was eluted with a 10 column volume linear gradient from Buffer A (20 mM phosphate, pH 7.2 with 15 % glycerol) to Buffer B (Buffer A plus 0.3 M NaCl). The flow rate was maintained throughout at 0.15 CV per minute. The eluent was monitored at 280 nm.

SDS-PAGE gels were run of the fractions and those containing the DiPEG conjugate were pooled and filtered though a 0.2 micron unit.

EXAMPLE 13

Biological Activity of Pegylated MGDF Molecules

A. PEG-9 - PEG-12 and PEG-14 - PEG-21

Platelet counts from mice treated with recombinant human MGDF were measured and the results are presented in FIG. 18. CHO-derived 22-353 (open diamond), unpegylated *E. coli* 22-184 (open circles) and

pegylated *E. coli* 22-184 (closed circles) MGDF at the concentrations indicated in the description of the figures above, were injected subcutaneously into normal Balb/c mice once daily for 5 days. Test bleeds from a small lateral cut in a tail vein were collected 24 hours after the last injection. Blood cell analyses were performed with a Sysmex electronic blood cell analyser (Baxter Diagnostics, Inc. Irvine, CA). Data are represented as the mean of determinations of 4 animals, +/- standard error of the mean. Other blood cell parameters such as total white blood cell counts or red blood cell counts were not affected by this treatment.

Additional forms of recombinant human MGDF were tested as above. Platelet counts from mice treated with either 50 ug/kg/day or 10 ug/kg/day of the indicated form of r-HuMGDF are shown in the following Table 10. Data are the mean of 4 animals and the standard errors are italicized.

Table 10

	50 ug/kg/day		10 ug/kg/day	
Form	Mean (n=4)	*sem*	Mean (n=4)	*sem*
CHO 22-353	4343	*309*	2571	*80*
E. coli 22-184	2021	*29*	1439	*18*
PEG 9	2728	*56*	2369	*34*
PEG 10	2431	*291*	1556	*126*
PEG 11	3778	*59*	1861	*73*
PEG 12	3885	*156*	1740	*88*
PEG 14	3567	*80*	2020	*63*
PEG 15	4402	*57*	2834	*99*
PEG 16	4511	*239*	3215	*11*
PEG 17	4140	*188*	3113	*261*
PEG 18	4586	*59*	2931	*129*
PEG 19	3980	*330*	4189	*80*
PEG 20	3942	*285*	3054	*339*
PEG 21	4195	*145*	4002	*91*
Baseline	939	*25*		

Key to Table 10

In each of the following, the MGDF molecule that was pegylated was *E. coli* derived MGDF-11 (amino acids 22-184, numbering from the beginning of the signal peptide or amino acids 1-163, numbering from the beginning of the mature protein), as described in the above Example 12:

Name	Pegylation	Avg. MW of PEG	Reactive PEG molecule for synthesis
PEG 9	polypegylated	6 kDa	NHS ester of MePEG
PEG 10	polypegylated	6 kDa	NHS ester of MePEG
PEG 11	polypegylated	20 kDa	NHS ester of MePEG
PEG 12	polypegylated	50 kDa	NHS ester of MePEG
PEG 14	monopegylated	6 kDa	Aldehyde of MePEG
PEG 15	monopegylated	12 kDa	Aldehyde of MePEG
PEG 16	monopegylated	20 kDa	Aldehyde of MePEG
PEG 17	monopegylated	25 kDa	Aldehyde of MePEG
PEG 18	polypegylated	6 kDa	Aldehyde of MePEG
PEG 19	polypegylated	12 kDa	Aldehyde of MePEG
PEG 20	polypegylated	20 kDa	Aldehyde of MePEG
PEG 21	polypegylated	25 kDa	Aldehyde of MePEG

The baseline counts are in normal animals without administration of any materials.

It is clear that pegylation of recombinant human MGDF does not adversely affect the ability of the molecule to increase platelet counts in recipient animals, and may in fact increase the activity of the *E. coli* product 22-184 to be as great or greater than that seen with the CHO-derived 22-353 molecule.

B. PEG-22

Results with PEG-22 are presented in FIG. 24. Notably, normalization of platelet counts with PEG-22 occurred several days sooner than with full-length CHO derived MGDF, PEG-16, or PEG-17.

EXAMPLE 14

Expression of recombinant human MGDF [1-163] in *E. coli*

To express r-HuMGDF in *E.coli*, the sequence encoding the first 163 amino acids of the mature protein was chemically synthesized utilizing optimal *E.coli* codons. Additionally, DNA sequences encoding the amino acids Methionine and Lysine were added to the 5' end of the gene. Therefore, the r-HuMGDF protein encoded by this sequence is 165 amino acids in length beginning with Met-Lys. The sequence of this gene is set forth in FIG. 25.

The synthesis of the r-HuMGDF (1-163) gene was accomplished in several steps. First, complementary oligonucleotides (60-70bp in length) representing adjoining fragments of the gene were chemically synthesized utilizing optimal *E.coli* codons. During this synthesis, codons for the amino acids Methionine and Lysine were placed at the 5' end of the mature gene, and a stop codon was placed at the 3' end of the gene. In addition, cutting sites for the restriction enzymes *Xba*I and *Hind*III were placed at the extreme 5' and 3' ends of the gene respectively, and a synthetic ribosome binding site was placed an appropriate distance upstream of the initiating Methionine. Second, the complementary oligonucleotides for each gene fragment were annealed. Third, these individual synthetic gene fragments were amplified using the Polymerase Chain Reaction. Fourth, amplified fragments were then sub-cloned into an appropriate vector. Fifth, the sequences of the sub-cloned fragments were verified. Sixth, the individual fragments were ligated together and sub-cloned into an appropriate vector, reconstructing the full-length r-HuMGDF (1-163) gene. Finally, the sequence of the reconstructed gene was verified.

The synthetic r-HuMGDF gene fragment, flanked by *Xba*I and *Hind*III restriction sites at the 5' and 3' ends respectively, contains a ribosome binding site, the ATG start codon, the sequence encoding the mature Met-Lys r-HuMGDF protein, and the stop codon.

The above fragment was cloned into the *Xba*I and *Hind*III sites of the lactose-inducible expression vector pAMG11. The pAMG11 vector is a low-copy-number plasmid with a pR100-derived origin of replication. The expression plasmid pAMG11 can be derived from the plasmid pCFM1656 (ATCC# 69576, deposited February 24, 1994) by making a series of site directed base changes by PCR overlapping oligo mutagenesis. Starting with the BglII site (plasmid bp # 180) immediately 5' to the plasmid replication promoter P_{copB} and proceeding toward the plasmid replication genes, the base pair changes are as follows:

pAMG11 bp #	bp in pCFM1656	bp changed to in pAMG11
# 204	T/A	C/G
# 428	A/T	G/C
# 509	G/C	A/T
# 617	- -	insert two G/C bp
# 679	G/C	T/A
# 980	T/A	C/G
# 994	G/C	A/T
# 1004	A/T	C/G
# 1007	C/G	T/A
# 1028	A/T	T/A
# 1047	C/G	T/A
# 1178	G/C	T/A
# 1466	G/C	T/A
# 2028	G/C	bp deletion
# 2187	C/G	T/A
# 2480	A/T	T/A
# 2499-2502	AGTG TCAC	GTCA CAGT
# 2642	TCCGAGC AGGCTCG	bp deletion
# 3435	G/C	A/T
# 3446	G/C	A/T
# 3643	A/T	T/A
# 4489-4512	- -	insert bps GAGCTCACTAGTGTCGACCTGCAG CTCGAGTGATCACAGCTGGACGTC

(SEQ ID NOS: 30, 31)

and by substituting the DNA sequence between the unique AatII and ClaI restriction sites with the following oligonucleotide:


```
AatII (#4358)
5'     CTCATAATTTTTAAAAAATTCATTTGACAAATGCTAAAATTCTT-
3' TGCAGAGTATTAAAAATTTTTTAAGTAAACTGTTTACGATTTTAAGAA-

-GATTAATATTCTCAATTGTGAGCGCTCACAATTTAT   3'
-CTAATTATAAGAGTTAACACTCGCGAGTGTTAAATAGC 5'
                                     ClaI (#4438)
```

(SEQ ID NOS: 32, 33)

Expression of r-HuMGDF, cloned into pAMG11, is driven by a synthetic lactose-inducible promoter, such as Ps4, which has the following sequence:

```
5'  GACGTCTCATAATTTTTAAAAAATTCATTTGACAAATGCTAAA-
     -ATTCTTGATTAATATTCTCAATTGTGAGCGCTCACAATTTATCGAT  3'.
```

(SEQ ID NO: 34)

The Ps4 promoter is repressed by the lactose repressor (LacI), the product of the *E.coli lacI* gene.

The pAMG11-r-HuMGDF plasmid was subsequently transformed into an *E.coli* K-12 strain containing the *lacI*^{q} allele. The *lacI*^{q} allele is a mutation within the *lacI* promoter which increases the expression of LacI, and results in a more stringent control of protein expression from the Ps4 promoter. Therefore, in this strain, in the absence of lactose, expression of r-HuMGDF is repressed by LacI. Upon the addition of lactose, LacI protein binding to the operator site on the Ps4 promoter is reduced, and transcription of r-HuMGDF from Ps4 is initiated. The *E. coli* host cell employed in this example is deposited under ATCC #69717, as of November 30, 1994.

The *E. coli* host ATCC # 69717 was transformed

with the pAMG11-r-HuMGDF plasmid and was grown according to the following fermentation description. The *E. coli* strain is inoculated into Luria broth and then incubated at 30°C for approximately 12 hours. The cells are then aseptically transferred into a fermentor that contains the batch medium (20 g/L yeast extract; 3.4 g/L citric acid; 15 g/L K_2HPO_4; 15 ml Dow P2000; 5 g/L glucose; 1 g/L $MgSO_4.7H_2O$; 5.5 ml/L trace metals; 5.5 ml/L vitamins). The batch phase of the process continues until the culture reaches an optical density of 5.0 ± 1.0 at 600 nm. The fed-batch phase is then begun with the initiation of the first feed medium (700 g/L glucose; 6.75 g/L $MgSO_4.7H_2O$). The feed rate is adjusted every 2 hours per an established schedule. The initiation of the second feed medium (129 g/L trypticase peptone; 258 g/L yeast extract) begins when the culture reaches an optical density of 20-25 at 600 nm. The second feed medium is maintained at a constant flow rate while the first feed medium continues to be adjusted. The temperature during the entire fermentation is maintained at approximately 30°C. The culture is maintained at about pH 7 with the addition of acid and base as necessary. The desired dissolved oxygen level is maintained by adjusting the agitation and air-input and oxygen-input rates in the fermentor. When the optical density of the culture reaches 57-63 at 600 nm addition of the third feed medium is initiated. The third feed medium (300 g/L lactose) is introduced to the fermentor at a constant flow rate; addition of the first feed medium is discontinued and the second feed medium flow rate is changed to a new constant rate. The fermentation lasts approximately ten hours after initiation of the third feed medium. At the end of the fermentation, the culture is chilled to 15 ± 5°C. The cells are harvested by centrifugation. The resulting paste is packaged and stored at < -60°C.

Purification of recombinant MGDF produced in *E. coli* as described above was carried out as follows. One thousand eight hundred grams of cell paste was suspended in about 18 liters of 10 mM EDTA and passed through a high pressure homogenizer at 15,000 psi. The broken cell suspension was centrifuged and the pellet was resuspended in 10 liter of 10 mM EDTA. The suspension was centrifuged and 200 g pellet was solubilized in 2 liter of 10 mM Tris, 8M Guanidine hydrochloride, 10 mm DTT, 5 mM EDTA, pH 8.7. This solution was slowly diluted into 200 liters of 10 mM CAPS, 3 M urea, 30% glycerol, 3mM cystamine, 1mM cysteine, pH 10.5.

The diluted solution was stirred slowly for 16 hr at room temperature and the pH was adjusted to 6.8. The pH adjusted solution was clarified and applied to a 2 liter CM Sepharose column equilibrated with 10 mM sodium phosphate, 1.5 M urea, 15% glycerol, pH 6.8. After loading, the column was washed with 10 mM sodium phosphate, 15% glycerol, pH 7.2. MGDF was eluted with a gradient of 0 to 0.5 M sodium chloride, 10 mM sodium phosphate, pH 7.2.

The CM eluate was concentrated and buffer exchanged with 10 mM sodium phosphate pH 6.5 with a 10,000 molecular weight cut off membrane. The concentrated solution, at about 2 mg per ml, was treated with cathepsin C (500 to 1 molar ratio) for 90 minutes at ambient temperature.

The solution was then loaded to a 1.2 liter SP High Performance Sepharose column equilibrated with 10 mM sodium phosphate, 15% glycerol, pH 7.2. After loading, MGDF was eluted with a gradient of 0.1 to 0.25 M sodium chloride, 10 mM sodium phosphate pH 7.2.

Ammonium sulfate was added to 0.6 M to the eluate from the SP High Performance column. The eluate was loaded to a 1.6 liter Phenyl Toyopearl column equilibrated with 10 mM sodium phosphate, 0.6 M ammonium sulfate, pH 7.2. The MGDF peak was eluted with a gradient of 0.6 to 0 M ammonium sulfate, 10 mM sodium phosphate, pH 7.2.

The Phenyl Toyopearl eluate was concentrated and buffer exchanged with a 10,000 molecular weight cut off membrane into 10 mM Tris, 5% sorbitol, pH 7.5.

EXAMPLE 15

In vivo, biological properties of r-HuMGDF (*E. coli* 1-163)

r-HuMGDF (*E. coli* 1-163), prepared as described in Example 14 above, was evaluated in rodents for biological efficacy. Normal, female Balb/c mice where injected subcutaneously for 5 consecutive days with increasing doses of r-HuMGDF. Doses ranged from 15 ug/kg/day to 1500 ug/kg/day. Twenty four hours after the last injection, blood cell counts were measured using an electronic cell counter (Sysmex, Baxter). A linear increase in platelet counts was observed with logarithmically increasing concentrations of the cytokine. Platelet counts increased to 300% of baseline values with 1500 ug/kg/day in this system. Other blood cell parameters were not affected with this treatment, such as white or red blood cell counts, or hematocrit.

Platelets were harvested from rats injected subcutaneously with 300 ug/kg/day r-HuMGDF (*E. coli* 1-163) for 6 days and evaluated for the ability to aggregate in response to ADP. The data indicate that platelets from treated animals are virtually indistinguishable from platelets from control animals in

that both populations are equivalently sensitive to the platelet agonist, ADP.

r-HuMGDF was also evaluated for the ability to abrogate the thrombocytopenia associated with chemotherapy and/or irradiation. Carboplatin, a chemotherapeutic which causes profound thrombocytopenia in humans, was used in these studies. Balb/c mice were injected subcutaneously with 1.25 mg carboplatin at the start of the study. After 24 hours, mice were injected daily with 100 ug/kg/day r-HuMGDF (*E. coli* 1-163), or excipient, for the remainder of the study. By Day 9, platelet counts dropped to roughly 15% of normal in excipient-treated mice but remained at baseline levels in mice treated with r-HuMGDF (see FIG. 20). For the irradiation studies, mice were subjected to a single dose of 500 rads of gamma-irradiation (Cesium source). This is a sublethal dose which results in a 90% reduction of platelet counts by Day 11. Platelet counts do not return to normal values until Day 21. When r-HuMGDF (*E. coli* 1-163) was administered once daily (100 ug/kg/day) to irradiated mice from Day 1 through Day 20, the drop in platelet counts was less severe and the return to baseline levels more rapid then mice treated with excipient (FIG. 21). In order to test r-HuMGDF in a model of extreme and prolonged thrombocytopenia, carboplatin and irradiation were applied in combination (FIG. 22). In this circumstance, platelet counts dropped to extremely low levels, (3-5% of normal), and most of the animals (7/8) did not survive this treatment. However, when these animals were treated daily with subcutaneous injections of r-HuMGDF at 100 ug/kg/day for the length of the study, thrombocytopenia was significantly abrogated, the return to baseline counts was more rapid, and all of the r-HuMGDF-treated animals (8/8) survived.

r-HuMGDF was also evaluated in rhesus monkeys.

Normal rhesus monkeys were subcutaneously injected with either 2.5 or 25 ug/kg/day for 10 days (Day 0-9). In the lower dose group, platelet counts increased by 400% at Day 12 and in the higher dose group they increased by 700%, also at Day 12. After the injections stopped, platelet counts returned to normal by Day 25-30. White blood cell counts and red blood cell counts were not affected by this treatment.

r-HuMGDF (*E. coli* 1-163) was also tested in a primate model of severe thrombocytopenia (FIG. 23). Animals were subjected to irradiation (700 rads, Cobalt source) which resulted in a reduction of platelet counts to 1-2% of normal by Day 15. By Day 35-40, platelet counts returned to normal. In contrast, the platelet counts in irradiated animals treated daily with r-HuMGDF (25 ug/kg/day) dropped to only 10% of normal and on average did not go below 20,000/ul, the trigger point for platelet transfusions in thrombocytopenic humans. The return to baseline counts was also more rapid in the r-HuMGDF-treated animals, occurring by Day 20.

These in vivo data from both rodent and primate studies fully support the concept that r-HuMGDF (*E. coli* 1-163) is a potent therapeutic agent with the capacity to significantly affect clinically relevant thrombocytopenias.

EXAMPLE 16

<u>Method for CHO Cell Culture Production of r-HuMGDF 1-332</u>

Glycosylated r-Hu MGDF 1-332 is produced from transfected Chinese Hamster Ovary cells expressing a cDNA for MGDF 1-332 under a suitable promoter and linked to a gene coding for the amplifiable selection marker, DHFR. A suitable promoter for expression of MGDF in CHO cells is SRα. *See* Mol. Cell. Biol. 8: 466-472 (1988)

and WO 91/13160 (1991). A suitable vector for expression of MGDF in CHO cells is pDSRα2. *See* WO 90/14363 (1990). Exemplary CHO cell lines can produce secreted MGDF in the range of 10-20 mg/L in standard cell culture media, but may be increased to 25 to ≥ 100 mg/L. To produce MGDF with a typical cell line, a culture can be expanded by passaging in suspension or in tissue culture vessels in adherent growth mode using medium comprised of equal proportions Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (DMEM/F12, Gibco) supplemented with 5 to 10% Fetal Bovine Serum (FBS) or dialyzed fetal bovine serum and methotrexate (MTX) (if necessary; typical concentration of MTX is 200-600 nM) to maintain selection pressure. This media should be supplemented with extra non-essential amino acids (NEAA's) and glutamine. Suspension cultures can propagate readily between inoculation (splitting) densities of 1-4 x 10^5 cells/mL and maximal densities of ~1 x 10^6 cells/mL at which point the cultures are expanded by dilution into larger volumes with initial cell densities at the specified splitting densities.

To produce MGDF in roller bottles, a suitable volume and cellular density of suspension culture must be generated using either magnetically stirred spinner vessels placed in a temperature controlled environment (37 ± 1°C), or an instrumented, controlled, stirred-tank bioreactor system. Roller bottles (such as 850 cm^2 Falcon roller bottles) should be seeded at initial densities of 1.5 to 3 x 10^7 cells per bottle and supplemented with additional growth medium (DMEM/F12 with 5-10% FBS, 1X NEAA and 1X L-glutamine) in an amount suitable to generate a confluent monolayer in 3-4 days (150-300 mL per bottle). The growth medium should be suitably buffered with sodium bicarbonate to a pH of 6.9 to 7.2 in equilibrium with carbon dioxide at a partial pressure of 60 to 90 mm Hg. Bottles should be gassed

with 10% CO_2/air and incubated on roller racks (~1 rpm) at 37 ± 1°C for 3-4 days. At confluence, the roller bottles should be shifted to serum-free production medium by pouring or aspirating the growth medium; washing the bottles with an isotonic buffer such as Dulbecco's Phosphate Buffered Saline (D-PBS), 50-100 mL per bottle; then adding an appropriate volume of bicarbonate-buffered, serum-free DMEM/F12 (1:1) (200 - 300 mL per bottle) supplemented with NEAA's and L-glutamine and with copper sulfate to minimize covalent aggregation (1-20 µM). Bottles should be gassed with 10% CO_2/air and incubated for 6 ± 1 days at 37 ± 1°C on roller racks (~1 rpm), or until metabolic activity has driven the glucose level to below 0.5 g/L and/or the pH level below 6.6. The conditioned medium should be harvested by pouring or aspirating from the bottles and replaced with fresh, serum-free production medium, as described above, for additional harvests. This can proceed until the cells can no longer sustain serum-free production and slough off of the roller bottles.

Harvested conditioned medium can be processed for purification by dead-end microfiltration through 0.45 µm and/or 0.2 µm filters (Sartorius Sartobran pH or Pall). Filtered conditioned medium should be chilled to 4°C, then either stored temporarily at 4°C, or immediately concentrated and dialyzed to low ionic strength using a cross-flow, ultrafiltration system (i.e. Filtron YM-50). Ultrafiltration and diafiltration should occur at 4°C to minimize protein degradation. Conditioned medium should be dialyzed with a buffered aqueous solution (i.e. 10 mM potassium phosphate, pH 6.8) prior to chromatographic purification steps.

Product quality in conditioned medium can be best monitored using non-reducing SDS-PAGE Western blots which can reveal the relative amounts of aggregated, monomeric, and proteolytically degraded MGDF in the

samples.

Another method for producing MGDF from CHO cells would be to adapt a cell line expressing MGDF to a serum-free medium such as Gibco S-SFM II. Cells can be adapted by serial passaging in medium containing minimal or no serum supplements. If a cell line is found to grow sustainably in such a medium while producing adequate amounts of secreted MGDF, production can proceed by scaling up an inoculum culture via serial passaging in increasingly larger culture volumes, then inoculating a suitable production vessel (an instrumented, controlled, stirred-tank bioreactor) and allowing the culture to proliferate to its maximal viable density under optimal growth conditions (pH, nutrients, temperature, oxygen, shear). At the optimal production point (as determined experimentally by measuring product quantity and quality) the culture can be harvested from the bioreactor, and the cells can be removed from the conditioned medium by micron-scale depth filtration or sub-micron cross-flow microfiltration. If depth filtration is used the medium should be further clarified by sub-micron dead-end filtration prior to concentration and dialysis as described above.

* * *

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art in light of the above description. Therefore, it is intended that the appended claims cover all such variations coming within the scope of the invention as claimed.

Additionally, the publications and other materials cited to illuminate the background of the invention, and in particular cases to provide additional details concerning its practice, are herein incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Bartley, Timothy D.
Bogenberger, Jakob M.
Bosselman, Robert A.
Hunt, Pamela
Kinstler, Olaf B.
Samal, Babru B.

(ii) TITLE OF INVENTION: Compositions and Methods for Stimulating Megakaryocyte Growth and Differentiation

(iii) NUMBER OF SEQUENCES: 34

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Amgen Inc.
(B) STREET: 1840 Dehavilland Drive
(C) CITY: Thousand Oaks
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 91320-1789

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Cook, Robert R.
(C) REFERENCE/DOCKET NUMBER: A-290-C

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ala Pro Pro Ala Xaa Asp Pro Arg Leu Leu Asn Lys Met Leu Arg Asp
1               5                   10                  15
```

Ser His Val Leu His Xaa Arg Leu Xaa Gln Xaa Pro Asp Ile Tyr
20 25 30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Pro Pro Ala Xaa Asp Pro Arg Leu Leu Asn Lys Met Leu Arg Asp
1 5 10 15

Ser His Val Leu His
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Gln Lys Glu Gln Thr Lys Ala Gln Asp Val Leu Gly Ala Val Ala
1 5 10 15

Leu

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCNCCNCCNG CNTGYGA 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCARTGYAAC ACRTGNGART C 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp
1 5 10 15

Ser His Val Leu His
20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTACGCGTTC TAGANNNNNN T 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTTTACTGA GGACTCGGAG G 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTCGGCCGGA TAGGCCTTTT TTTTTTTTTT 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTCGGCCGGA TAGGCCTTTT TTTTTTTTT 29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGCGACCTCC GAGTCCTCAG 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGTCCTCAG TAAACTGCTT CGT 23

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAGTCACGA AGCAGTTTAC 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCTTTACTTC TAGGCCTG 18

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGGTCACAA GCAGGAGGA 19

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCATAGTCC GGGACGTCG 19

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCCTCCTGCT TGTGACCTC 19

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCAGGAAGGA TTCAGGGGA 19

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAACAAGTCG ACCGCCAGCC AGACACCCCG 30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCCGGATAG GCCACTCNNN NNNT 24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCARTGYAAN ACRTGNGART C 21

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
TTGGTGTGCA CTTGTG                                          16
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
CACAAGTGCA CACCAACCCC                                      20
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1342 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 99..1094

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
CAGGGAGCCA CGCCAGCCAA GACACCCCGG CCAGAATGGA GCTGACTGAA TTGCTCCTCG     60

TGGTCATGCT TCTCCTAACT GCAAGGCTAA CGCTGTCC AGC CCG GCT CCT CCT        113
                                          Ser Pro Ala Pro Pro
                                            1               5
```



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GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC      161
Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val
                 10                  15                  20

CTT CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA      209
Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr
             25                  30                  35

CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC      257
Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr
         40                  45                  50

CAG ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT      305
Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala Val Thr Leu
     55                  60                  65

CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC      353
Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys
 70                  75                  80                  85

CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT      401
Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu
                 90                  95                 100

GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG      449
Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg
            105                 110                 115

ACC ACA GCT CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC      497
Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His
        120                 125                 130

CTG CTC CGA GGA AAG GTG CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC      545
Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr
    135                 140                 145

CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA GCT GTC CCC AGC AGA ACC      593
Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr
150                 155                 160                 165

TCT CTA GTC CTC ACA CTG AAC GAG CTC CCA AAC AGG ACT TCT GGA TTG      641
Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu
                170                 175                 180

TTG GAG ACA AAC TTC ACT GCC TCA GCC AGA ACT ACT GGC TCT GGG CTT      689
Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu
            185                 190                 195

CTG AAG TGG CAG CAG GGA TTC AGA GCC AAG ATT CCT GGT CTG CTG AAC      737
Leu Lys Trp Gln Gln Gly Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn
        200                 205                 210

CAA ACC TCC AGG TCC CTG GAC CAA ATC CCC GGA TAC CTG AAC AGG ATA      785
Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile
    215                 220                 225
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```
CAC GAA CTC TTG AAT GGA ACT CGT GGA CTC TTT CCT GGA CCC TCA CGC        833
His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg
230                 235                 240                 245

AGG ACC CTA GGA GCC CCG GAC ATT TCC TCA GGA ACA TCA GAC ACA GGC        881
Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly
            250                 255                 260

TCC CTG CCA CCC AAC CTC CAG CCT GGA TAT TCT CCT TCC CCA ACC CAT        929
Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr Ser Pro Ser Pro Thr His
        265                 270                 275

CCT CCT ACT GGA CAG TAT ACG CTC TTC CCT CTT CCA CCC ACC TTG CCC        977
Pro Pro Thr Gly Gln Tyr Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro
    280                 285                 290

ACC CCT GTG GTC CAG CTC CAC CCC CTG CTT CCT GAC CCT TCT GCT CCA       1025
Thr Pro Val Val Gln Leu His Pro Leu Leu Pro Asp Pro Ser Ala Pro
    295                 300                 305

ACG CCC ACC CCT ACC AGC CCT CTT CTA AAC ACA TCC TAC ACC CAC TCC       1073
Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn Thr Ser Tyr Thr His Ser
310                 315                 320                 325

CAG AAT CTG TCT CAG GAA GGG TAAGGTTCTC AGACACTGCC GACATCAGCA          1124
Gln Asn Leu Ser Gln Glu Gly
            330

TTGTCTCGTG TACAGCTCCC TTCCCTGCAG GGCGCCCCTG GGAGACAACT GGACAAGATT     1184

TCCTACTTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA TACACAGGAC TGAAAAGGGA     1244

ATCATTTTTC ACTGTACATT ATAAACCTTC AGAAGCTATT TTTTTAAGCT ATCAGCAATA     1304

CTCATCAGAG CAGCTAGCTC TTTGGTCTAT TTTCTGCA                             1342
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 332 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu
 1               5                  10                  15

Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val
            20                  25                  30

His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu
        35                  40                  45
```



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Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu
    50                  55                  60

Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln
65                  70                  75                  80

Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln
                85                  90                  95

Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu
            100                 105                 110

Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe
        115                 120                 125

Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu
    130                 135                 140

Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala
145                 150                 155                 160

Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn
                165                 170                 175

Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg Thr
            180                 185                 190

Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly Phe Arg Ala Lys Ile
        195                 200                 205

Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly
    210                 215                 220

Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe
225                 230                 235                 240

Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser Gly
                245                 250                 255

Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr Ser
            260                 265                 270

Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu Phe Pro Leu
        275                 280                 285

Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu His Pro Leu Leu Pro
    290                 295                 300

Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn Thr
305                 310                 315                 320

Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu Gly
                325                 330
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1342 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 99..621

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
CAGGGAGCCA CGCCAGCCAA GACACCCCGG CCAGAATGGA GCTGACTGAA TTGCTCCTCG        60

TGGTCATGCT TCTCCTAACT GCAAGGCTAA CGCTGTCC AGC CCG GCT CCT CCT           113
                                          Ser Pro Ala Pro Pro
                                            1               5

GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC         161
Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val
                 10                  15                  20

CTT CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA         209
Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr
             25                  30                  35

CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC         257
Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr
         40                  45                  50

CAG ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT         305
Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala Val Thr Leu
     55                  60                  65

CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC         353
Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys
 70                  75                  80                  85

CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT         401
Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu
                 90                  95                 100

GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG         449
Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg
            105                 110                 115

ACC ACA GCT CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC         497
Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His
        120                 125                 130
```



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TG CTC CGA GGA AAG GTG CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC          545
Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr
    135                 140                 145

CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA GCT GTC CCC AGC AGA ACC         593
Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr
150                 155                 160                 165

TCT CTA GTC CTC ACA CTG AAC GAG CTC C CAAACAGGAC TTCTGGATTG             641
Ser Leu Val Leu Thr Leu Asn Glu Leu
                170

TTGGAGACAA ACTTCACTGC CTCAGCCAGA ACTACTGGCT CTGGGCTTCT GAAGTGGCAG       701

CAGGGATTCA GAGCCAAGAT TCCTGGTCTG CTGAACCAAA CCTCCAGGTC CCTGGACCAA       761

ATCCCCGGAT ACCTGAACAG GATACACGAA CTCTTGAATG GAACTCGTGG ACTCTTTCCT       821

GGACCCTCAC GCAGGACCCT AGGAGCCCCG GACATTTCCT CAGGAACATC AGACACAGGC       881

TCCCTGCCAC CCAACCTCCA GCCTGGATAT TCTCCTTCCC CAACCCATCC TCCTACTGGA       941

CAGTATACGC TCTTCCCTCT TCCACCCACC TTGCCCACCC CTGTGGTCCA GCTCCACCCC      1001

CTGCTTCCTG ACCCTTCTGC TCCAACGCCC ACCCCTACCA GCCCTCTTCT AAACACATCC      1061

TACACCCACT CCCAGAATCT GTCTCAGGAA GGGTAAGGTT CTCAGACACT GCCGACATCA      1121

GCATTGTCTC GTGTACAGCT CCCTTCCCTG CAGGGCGCCC CTGGGAGACA ACTGGACAAG      1181

ATTTCCTACT TTCTCCTGAA ACCCAAAGCC CTGGTAAAAG GGATACACAG GACTGAAAAG      1241

GGAATCATTT TTCACTGTAC ATTATAAACC TTCAGAAGCT ATTTTTTTAA GCTATCAGCA      1301

ATACTCATCA GAGCAGCTAG CTCTTTGGTC TATTTTCTGC A                          1342
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 174 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu
  1               5                  10                  15

Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val
             20                  25                  30

His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu
         35                  40                  45
```

```
Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu
 50                     55                  60

Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln
 65                 70                  75                  80

Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln
                 85                  90                  95

Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu
            100                 105                 110

Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe
        115                 120                 125

Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu
    130                 135                 140

Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala
145                 150                 155                 160

Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu
                165                 170
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 97..894

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
AGGGAGCCAC GCCAGCCAGA CACCCCGGCC AGAATGGAGC TGACTGAATT GCTCCTCGTG      60

GTCATGCTTC TCCTAACTGC AAGGCTAACG CTGTCC AGC CCG GCT CCT CCT GCT       114
                                        Ser Pro Ala Pro Pro Ala
                                          1               5

TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC CTT      162
Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu
             10                  15                  20

CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT      210
His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro
         25                  30                  35
```



```
GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC CAG      258
Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln
     40                  45                  50

ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT CTG      306
Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu
 55                  60                  65                  70

CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC      354
Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu
                 75                  80                  85

TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG      402
Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly
             90                  95                 100

GCC CTG CAG AGC CTC CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC      450
Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr
        105                 110                 115

ACA GCT CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG      498
Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu
    120                 125                 130

CTC CGA GGA AAG GAC TTC TGG ATT GTT GGA GAC AAA CTT CAC TGC CTC      546
Leu Arg Gly Lys Asp Phe Trp Ile Val Gly Asp Lys Leu His Cys Leu
135                 140                 145                 150

AGC CAG AAC TAC TGG CTC TGG GCT TCT GAA GTG GCA GCA GGG ATT CAG      594
Ser Gln Asn Tyr Trp Leu Trp Ala Ser Glu Val Ala Ala Gly Ile Gln
                155                 160                 165

AGC CAA GAT TCC TGG TCT GCT GAA CCA AAC CTC CAG GTC CCT GGA CCA      642
Ser Gln Asp Ser Trp Ser Ala Glu Pro Asn Leu Gln Val Pro Gly Pro
            170                 175                 180

AAT CCC CGG ATA CCT GAA CAG GAT ACA CGA ACT CTT GAA TGG AAC TCG      690
Asn Pro Arg Ile Pro Glu Gln Asp Thr Arg Thr Leu Glu Trp Asn Ser
        185                 190                 195

TGG ACT CTT TCC TGG ACC CTC ACG CAG GAC CCT AGG AGC CCC GGA CAT      738
Trp Thr Leu Ser Trp Thr Leu Thr Gln Asp Pro Arg Ser Pro Gly His
    200                 205                 210

TTC CTC AGG AAC ATC AGA CAC AGG CTC CCT GCC ACC CAA CCT CCA GCC      786
Phe Leu Arg Asn Ile Arg His Arg Leu Pro Ala Thr Gln Pro Pro Ala
215                 220                 225                 230

TGG ATA TTC TCC TTC CCC AAC CCA TCC TCC TAC TGG ACA GTA TAC GCT      834
Trp Ile Phe Ser Phe Pro Asn Pro Ser Ser Tyr Trp Thr Val Tyr Ala
                235                 240                 245

CTT CCC TCT TCC ACC CAC CTT GCC CAC CCC TGT GGT CCA GCT CCA CCC      882
Leu Pro Ser Ser Thr His Leu Ala His Pro Cys Gly Pro Ala Pro Pro
            250                 255                 260
```

```
CCT GCT TCC TGACCCTTCT GCTCCAACGC CCACCCCTAC CAGCCCTCTT              931
Pro Ala Ser
        265

CTAAACACAT CCTACACCCA CTCCCAGAAT CTGTCTCAGG AAGGGTAAGG TTCTCAGACA    991

CTGCCGACAT CAGCATTGTC TCGTGTACAG CTCCCTTCCC TGCAGGGCGC CCCTGGGAGA   1051

CAACTGGACA AGATTTCCTA CTTTCTCCTG AAACCCAAAG CCCTGGTAAA AGGGATACAC   1111

AGGACTGAAA AGGGAATCAT TTTTCACTGT ACATTATAAA CCTTCAGAAG CTA           1164
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 265 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu
 1               5                  10                  15

Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val
            20                  25                  30

His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu
        35                  40                  45

Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu
    50                  55                  60

Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln
65                  70                  75                  80

Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln
                85                  90                  95

Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu
            100                 105                 110

Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe
        115                 120                 125

Leu Ser Phe Gln His Leu Leu Arg Gly Lys Asp Phe Trp Ile Val Gly
    130                 135                 140

Asp Lys Leu His Cys Leu Ser Gln Asn Tyr Trp Leu Trp Ala Ser Glu
145                 150                 155                 160

Val Ala Ala Gly Ile Gln Ser Gln Asp Ser Trp Ser Ala Glu Pro Asn
                165                 170                 175
```



```
Leu Gln Val Pro Gly Pro Asn Pro Arg Ile Pro Glu Gln Asp Thr Arg
            180                 185                 190

Thr Leu Glu Trp Asn Ser Trp Thr Leu Ser Trp Thr Leu Thr Gln Asp
        195                 200                 205

Pro Arg Ser Pro Gly His Phe Leu Arg Asn Ile Arg His Arg Leu Pro
    210                 215                 220

Ala Thr Gln Pro Pro Ala Trp Ile Phe Ser Phe Pro Asn Pro Ser Ser
225                 230                 235                 240

Tyr Trp Thr Val Tyr Ala Leu Pro Ser Ser Thr His Leu Ala His Pro
                245                 250                 255

Cys Gly Pro Ala Pro Pro Pro Ala Ser
            260                 265
```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
GAGCTCACTA GTGTCGACCT GCAG                                            24
```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
CTGCAGGTCG ACACTAGTGA GCTC                                            24
```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTCATAATTT TTAAAAAATT CATTTGACAA ATGCTAAAAT TCTTGATTAA TATTCTCAAT 60

TGTGAGCGCT CACAATTTAT 80

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CGATAAATTG TGAGCGCTCA CAATTGAGAA TATTAATCAA GAATTTTAGC ATTTGTCAAA 60

TGAATTTTTT AAAAATTATG AGACGT 86

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GACGTCTCAT AATTTTTAAA AAATTCATTT GACAAATGCT AAAATTCTTG ATTAATATTC 60

TCAATTGTGA GCGCTCACAA TTTATCGAT 89

WHAT IS CLAIMED IS:

1. A human MGDF polypeptide that specifically promotes the growth and development of human megakaryocytes, substantially free from other human proteins.

2. A polypeptide according to Claim 1, wherein said polypeptide comprises amino acids 22-172 of FIG. 11.

3. A polypeptide according to Claim 2, wherein said polypeptide comprises amino acids 22-195 of FIG. 11.

4. A polypeptide according to Claim 3, having the amino acid sequence of MGDF-2.

5. A polypeptide according to Claim 1, wherein said polypeptide comprises amino acids 22-353 of FIG. 11.

6. A polypeptide according to Claim 5, having the amino acid sequence of MGDF-1.

7. A polypeptide according to Claim 2, which has an amino acid sequence of a member selected from the group consisting of MGDF-4, MGDF-5, MGDF-6, MGDF-7, and MGDF-8.

8. A polypeptide according to Claim 1, wherein said polypeptide comprises amino acids 22-184 of FIG. 11.

9. A polypeptide according to Claim 1, wherein said polypeptide further comprises the sequence Met-Lys at the N-terminus thereof.

10. A polypeptide according to Claim 1, wherein said polypeptide comprises amino acids 22-184 of FIG. 11 and further comprises the sequence Met-Lys at the N-terminus thereof.

11. A polypeptide according to Claim 1, wherein said polypeptide comprises amino acids 22-353 of FIG. 11 and further comprises the sequence Met-Lys at the N-terminus thereof.

12. A polypeptide according to Claim 1, further comprising amino acids 1-21 of FIG. 11.

13. An isolated polynucleotide encoding a human MGDF polypeptide.

14. An isolated polynucleotide according to Claim 13, which encodes a human MGDF polypeptide according to any of Claims 1-12.

15. An isolated polynucleotide according to Claim 14, which is a DNA sequence.

16. A DNA sequence according to Claim 15, which is a cDNA sequence.

17. A cDNA sequence according to Claim 16, which has a sequence as shown in FIG 11 or 12.

18. A DNA vector comprising a DNA sequence according to Claim 15.

19. The vector of Claim 18 wherein said DNA sequence is operatively linked to an expression control DNA sequence.

20. A host cell stably transformed or transfected with a DNA sequence according to Claim 15.

21. A host cell according to Claim 20, which expresses said MGDF polypeptide encoded by said DNA sequence.

22. A method for producing a human MGDF polypeptide, said method comprising growing a host cell according to Claim 21 in a suitable nutrient medium and isolating said MGDF polypeptide from said cell or said nutrient medium.

23. A method for producing a human MGDF polypeptide according to Claim 22, wherein said host cell is an *E. coli* cell.

24. A method for producing a human MGDF polypeptide according to Claim 22, wherein said host cell is a CHO cell.

25. An antibody reactive with a human MGDF polypeptide according to any of Claims 1-12.

26. A monoclonal antibody according to Claim 25.

27. A recombinant antibody according to Claim 25.

28. A pharmaceutical composition comprising a human MGDF polypeptide according to any of Claims 1-12

in association with a pharmaceutically acceptable diluent, adjuvant, or carrier.

29. A pharmaceutical composition according to Claim 28 in aqueous solution.

30. A pharmaceutical composition according to Claim 28 in lyophilized form.

31. A method for treating a patient having a deficiency of a human MGDF polypeptide, which comprises administering an effective amount of a human MGDF polypeptide according to any of Claims 1-12 to said patient.

32. A method for treating a patient having a thrombocytopenic condition, which comprises administering an effective amount of a human MGDF polypeptide according to any of Claims 1-12 to said patient.

33. A method according to Claim 32, wherein said condition is selected from the group consisting of aplastic anemia, idiopathic thrombocytopenia, and thrombocytopenia resulting from drug or radiation treatment.

34. A method for increasing the number of mature megakaryocytes in a patient in need thereof, which comprises administering to said patient an effective amount of a human MGDF polypeptide according to any of Claims 1-12.

35. A method for increasing the number of platelets in a patient in need thereof, which comprises

administering to said patient an effective amount of a human MGDF polypeptide according to any of Claims 1-12.

36. An MGDF derivative comprising an MGDF polypeptide according to any of Claims 1-12 connected to at least one water soluble polymer.

37. An MGDF derivative of Claim 36, wherein said MGDF polypeptide is selected from the group consisting of MGDF-1, MGDF-2, MGDF-4, MGDF-11, MGDF-12, MGDF-13, MGDF-14, and MGDF-15.

38. An MGDF derivative of Claim 36, wherein said MGDF polypeptide is recombinantly produced in a bacterial cell.

39. An MGDF derivative of Claim 36, wherein said water soluble polymer is pharmaceutically acceptable.

40. An MGDF derivative of Claim 36, wherein said water soluble polymer is selected from the group consisting of dextran, poly(N-vinyl-pyrrolidone), polyethylene glycols, polypropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, polyvinyl alcohols and mixtures thereof.

41. An MGDF derivative of Claim 36, wherein said water soluble polymer is a polyethylene glycol.

42. An MGDF derivative according to Claim 41, wherein said polyethylene glycol is a monomethoxy-polyethylene glycol.

43. An MGDF derivative according to Claim 41, wherein said polyethylene glycol is attached to said MGDF polypeptide by an acyl or an alkyl linkage.

44. Pegylated MGDF polypeptide having an amino acid sequence according to any of Claims 1-12.

45. A pegylated MGDF polypeptide according to Claim 44, which has the amino acid sequence of amino acids 22-184 of FIG. 11.

46. A pegylated MGDF polypeptide according to Claim 45, wherein the polyethylene glycol group is attached to the N-terminus thereof.

47. A pegylated MGDF polypeptide according to Claim 46, wherein the polyethylene glycol group has an average molecular weight of 10 to 50 kilodaltons.

48. A pegylated MGDF polypeptide according to Claim 47, wherein said MGDF polypeptide is produced in *E. coli*.

49. Monopegylated MGDF polypeptide having an amino acid sequence according to any of Claims 1-12.

50. An MGDF derivative comprising an MGDF polypeptide according to any of Claims 1-12 covalently attached to two water soluble polymer molecules.

51. An MGDF derivative according to Claim 50, wherein said water soluble polymer molecules are both polyethylene glycols.

52. An MGDF derivative according to Claim 51, wherein said MGDF polypeptide comprises amino acids 22

to 184 of FIG. 11 and said polyethylene glycols have an average molecular weight of 5 to 25 kilodaltons.

53. A method for attaching a water soluble polymer to an MGDF polypeptide according to any of Claims 1-12, wherein said water soluble polymer has a single reactive aldehyde group, said method comprising:

(a) contacting said MGDF polypeptide with a water soluble polymer under reductive alkylation conditions, at a pH sufficiently acidic to allow the α-amino group at the amino terminus of said MGDF polypeptide to be reactive; and

(b) isolating said MGDF polypeptide attached to at least one water soluble polymer.

54. A method for attaching a water soluble polymer to an MGDF polypeptide according to any of Claims 1-12, wherein said water soluble polymer has a single reactive ester group, said method comprising:

(a) contacting said MGDF polypeptide with a water soluble polymer under conditions so that said MGDF polypeptide becomes attached to the water soluble polymer through an acyl linkage; and

(b) isolating an MGDF polypeptide attached to at least one water soluble polymer.

55. A method of Claim 53 or 54, wherein said polymer is pharmaceutically acceptable.

56. A method of Claim 53 or 54, wherein said water soluble polymer is selected from the group consisting of dextran, poly(N-vinyl-pyrrolidone), polyethylene glycols, polypropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohols.

57. A method of Claim 53 or 54, wherein said water soluble polymer is polyethylene glycol.

58. A method of Claim 53 wherein said pH is between about 3 and about 9.

59. A method of Claim 53, wherein said reductive alkylation conditions involve the use of sodium cyanoborohydride as a reducing agent.

60. A method for attaching a polyethylene glycol molecule to an MGDF polypeptide according to any of Claims 1-12, wherein said polyethylene glycol molecule has a single reactive aldehyde group, said method comprising:

(a) contacting said MGDF polypeptide with said polyethylene glycol molecule under reductive alkylation conditions, at a pH sufficiently acidic to allow the α-amino group at the amino terminus of said MGDF polypeptide to be reactive; and

(b) obtaining a pegylated MGDF polypeptide.

61. A method of Claim 60, wherein said polyethylene glycol molecule has a molecular weight of 2 to 100 kilodaltons.

62. A pegylated MGDF polypeptide product produced by the process of Claim 60.

63. A substantially homogeneous preparation of an MGDF polypeptide according to any of Claims 1-12 monopegylated at the α-amino group at the N-terminus of said MGDF polypeptide.

64. A preparation of Claim 63, wherein said MGDF polypeptide is monopegylated with a polyethylene glycol having an average molecular weight of 5 to 50 kilodaltons.

65. A pharmaceutical composition comprising a pegylated MGDF polypeptide according to any of Claims 41-49, 51 or 52 and a pharmaceutically acceptable diluent, adjuvant or carrier.

66. A pharmaceutical composition comprising: (a) a substantially homogeneous preparation of monopegylated MGDF polypeptide according to Claim 63, said monopegylated MGDF polypeptide consisting of a polyethylene glycol having a molecular weight of 5 to 50 kilodatons connected to an MGDF polypeptide solely at the N-terminus thereof via an alkyl linkage; and (b) a pharmaceutically acceptable diluent, adjuvant or carrier.

67. A mono-pegylated MGDF polypeptide comprising amino acids 22-184 of Figure 11.

68. A mono-pegylated MGDF polypeptide according to Claim 67, wherein the polyethylene glycol group is attached to the N-terminus of the polypeptide.

69. A mono-pegylated MGDF polypeptide according to Claim 68, wherein the polyethylene glycol group has an average molecular weight of 2 to 100 kilodaltons.

70. A mono-pegylated MGDF polypeptide according to Claim 67, 68 or 69, wherein the MGDF polypeptide has been produced in *E. coli*.

71. A method of attaching water soluble polyethylene glycol to an MGDF polypeptide comprising amino acids 22-184 of Figure 11, which method comprises contacting the MGDF polypeptide with the polyethylene glycol under conditions so that the MGDF polypeptide becomes attached to the polyethylene glycol to obtain a mono-pegylated MGDF polypeptide.

72. A method according to Claim 71, wherein the polyethylene glycol is attached to the MGDF polypeptide under reductive alkylation conditions at a pH sufficiently acidic to allow the α-amino group at the N-terminus of the MGDF polypeptide to be reactive.

73. A method according to Claim 71 or 72, wherein the MGDF polypeptide is mono-pegylated with a polyethylene glycol having an average molecular weight of 2 to 100 kilodaltons.

74. A method according to Claim 73, wherein the MGDF polypeptide is made by cleaving Met^{-2}-Lys^{-1} from a polypeptide obtained by expression in an *E. coli* cell of a DNA encoding a polypeptide comprising amino acids 22-184 of Figure 11 and the sequence of Met-Lys at the N-terminus thereof.

75. A method according to Claim 73, wherein the MGDF polypeptide is made by:

a. expressing in an *E. coli* cell a DNA encoding a polypeptide comprising amino acids 22-184 of Figure 11 and the sequence of Met-Lys at the N-terminus thereof,

b. isolating the expressed polypeptide, and

c. cleaving Met^{-2}-Lys^{-1} from the isolated polypeptide.

76. A pharmaceutical composition comprising mono-pegylated MGDF polypeptide according to Claim 69 and a pharmaceutically acceptable diluent, adjuvant or carrier.

77. A pharmaceutical composition according to Claim 76 in aqueous solution.

78. A pharmaceutical composition according to Claim 76 in lyophilized form.

79. A method for treating a patient having a deficiency of a human MGDF polypeptide, which comprises administering an effective amount of a mono-pegylated MGDF polypeptide according to Claim 69 to said patient.

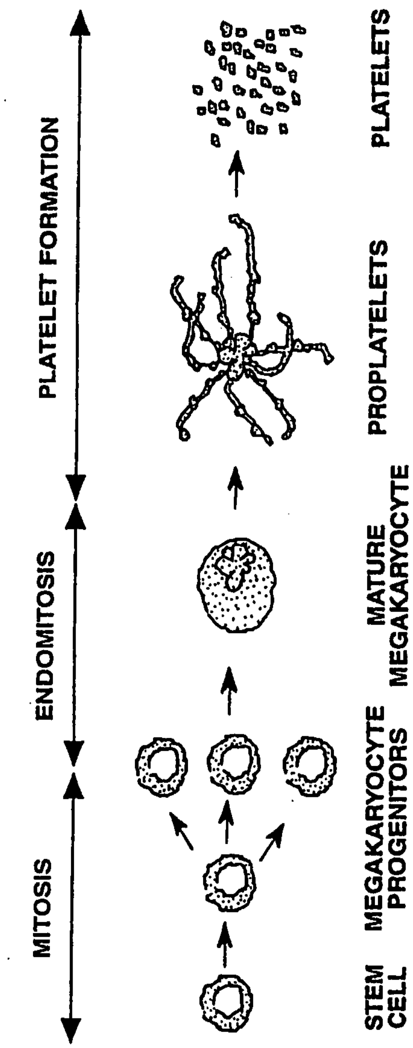
80. A method for treating a patient having a thrombocytopenic condition, which comprises administering an effective amount of a mono-pegylated MGDF polypeptide according to Claim 69 to said patient.

81. A method according to Claim 80, wherein said condition is selected from the group consisting of aplastic anemia, idiopathic thrombocytopenia, and thrombocytopenia resulting from drug or radiation treatment.

82. A method for increasing the number of mature megakaryocytes in a patient in need thereof, which comprises administering to said patient an effective amount of a mono-pegylated MGDF polypeptide according to Claim 69.

83. A method for increasing the number of platelets in a patient in need thereof, which comprises

administering to said patient an effective amount of a mono-pegylated MGDF polypeptide according to Claim 69.



Megakaryocyte and Platelet Development

FIG. 1

FIG. 2

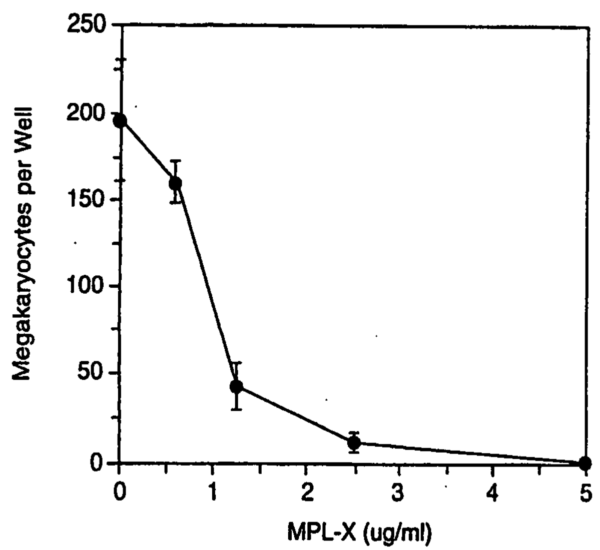

Megakaryocytes per Well
250
200
150
100
50
0
0
1
2
3
4
5
MPL-X (ug/ml)

FIG. 3

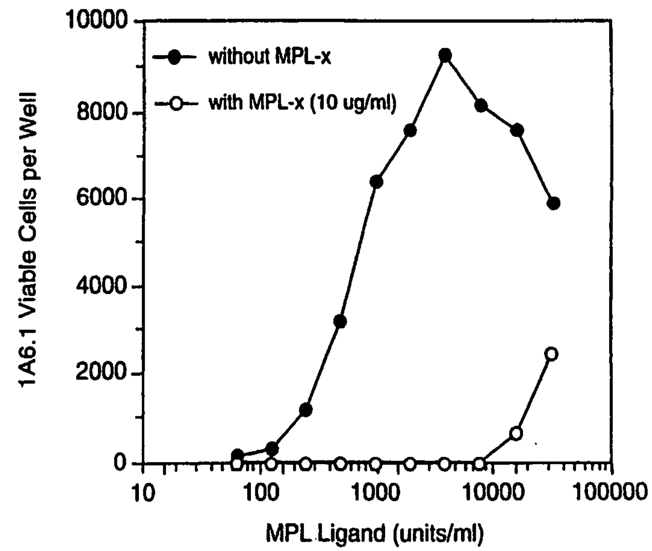

1A6.1 Viable Cells per Well
10000
8000
6000
4000
2000
0
without MPL-x
with MPL-x (10 ug/ml)
10
100
1000
10000
100000
MPL Ligand (units/ml)

FIG. 4

Purification of Mpl Ligand

Canine (irradiated) Plasma
↓
Wheat Germ Agglutinin (lectin) Affinity Chromatography
↓
Murine Mpl-X Receptor Affinity Chromatography
↓
Anion Exchange Chromatography
↓
Gel Filtration Chromatography (0.1% SDS)
↓
C4 Reversed Phase High Performance Liquid Chromatography
↓
Purified Mpl Ligand

FIG. 5A

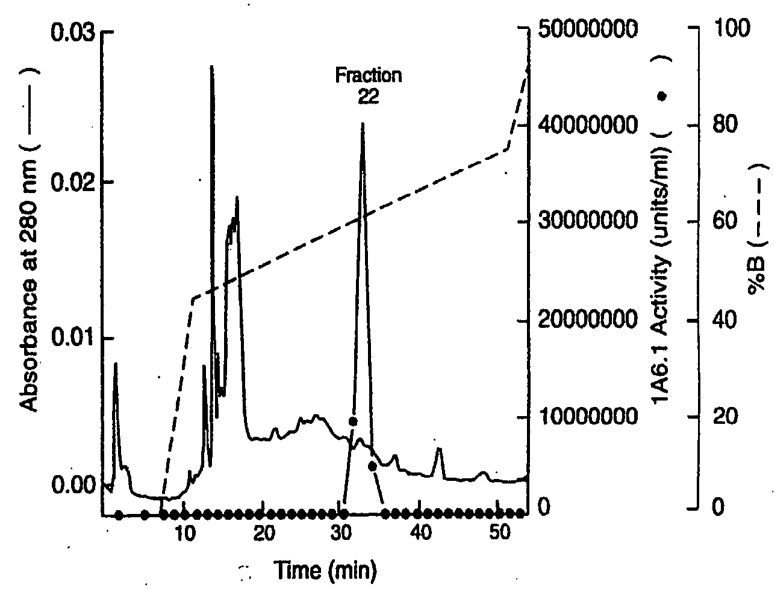
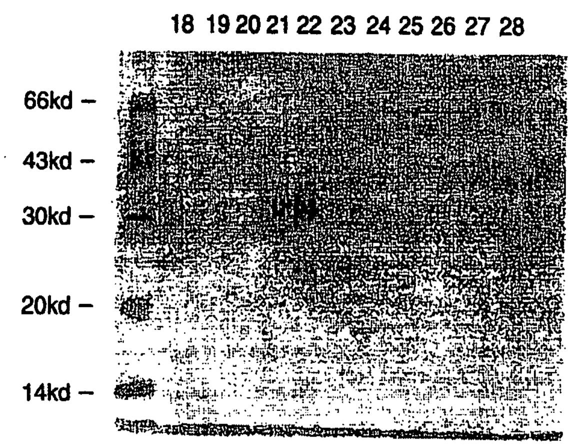
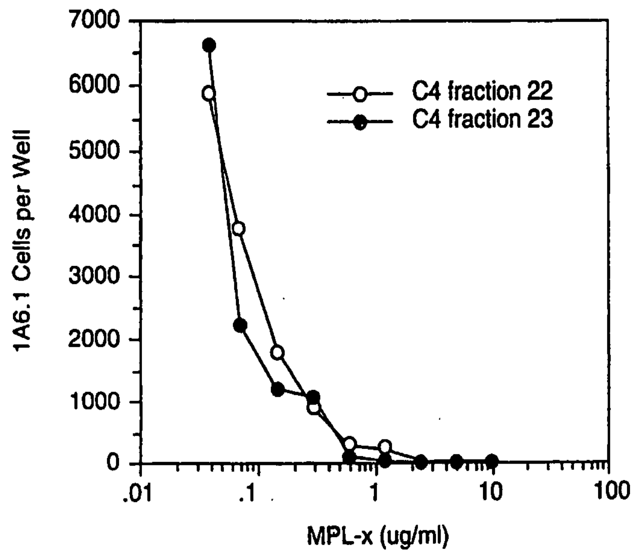


FIG. 5B



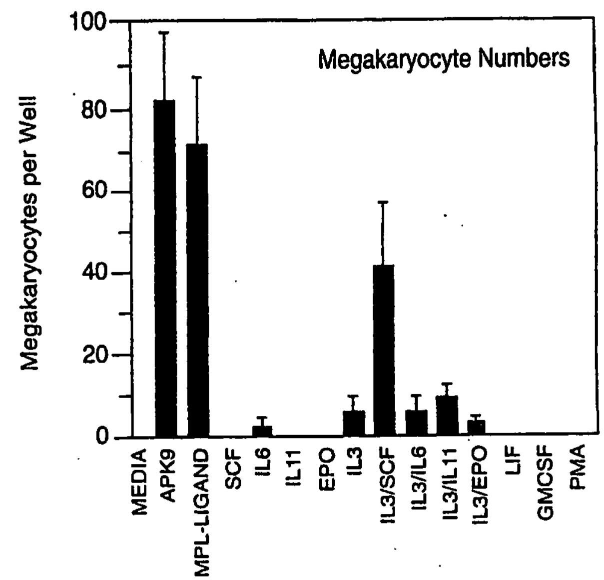
SDS-PAGE 14% NONREDUCING

FIG. 6



1A6.1 Cells per Well
7000
6000
5000
4000
3000
2000
1000
0
.01
.1
1
10
100
MPL-x (ug/ml)
C4 fraction 22
C4 fraction 23

FIG. 7



Megakaryocytes per Well
Megakaryocyte Numbers
100
80
60
40
20
0
MEDIA
APK9
MPL-LIGAND
SCF
IL6
IL11
EPO
IL3
IL3/SCF
IL3/IL6
IL3/IL11
IL3/EPO
LIF
GMCSF
PMA

FIG. 8

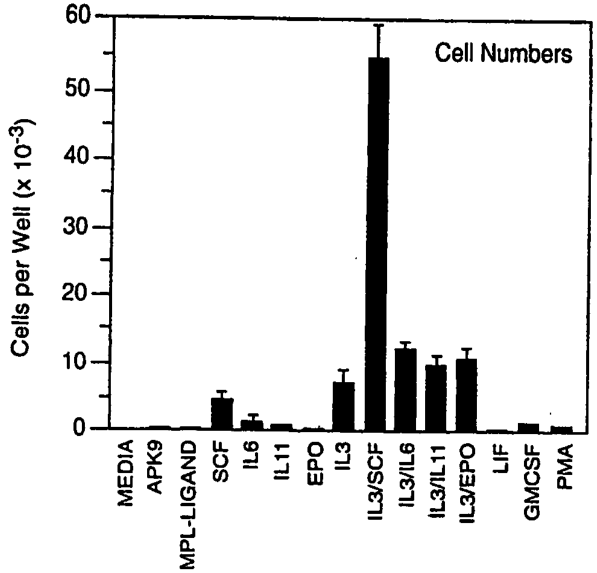

Cell Numbers
Cells per Well (x 10-3)
60
50
40
30
20
10
0
MEDIA
APK9
MPL-LIGAND
SCF
IL6
IL11
EPO
IL3
IL3/SCF
IL3/IL6
IL3/IL11
IL3/EPO
LIF
GMCSF
PMA

FIG. 9

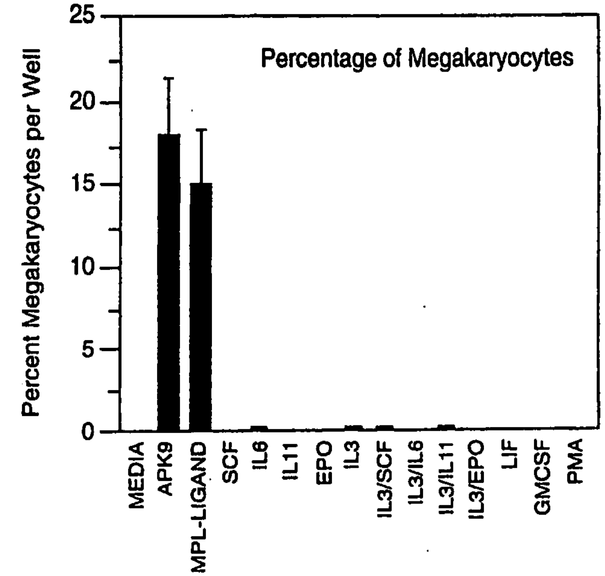

Percentage of Megakaryocytes
Percent Megakaryocytes per Well
25
20
15
10
5
0
MEDIA
APK9
MPL-LIGAND
SCF
IL6
IL11
EPO
IL3
IL3/SCF
IL3/IL6
IL3/IL11
IL3/EPO
LIF
GMCSF
PMA

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FIG. 10

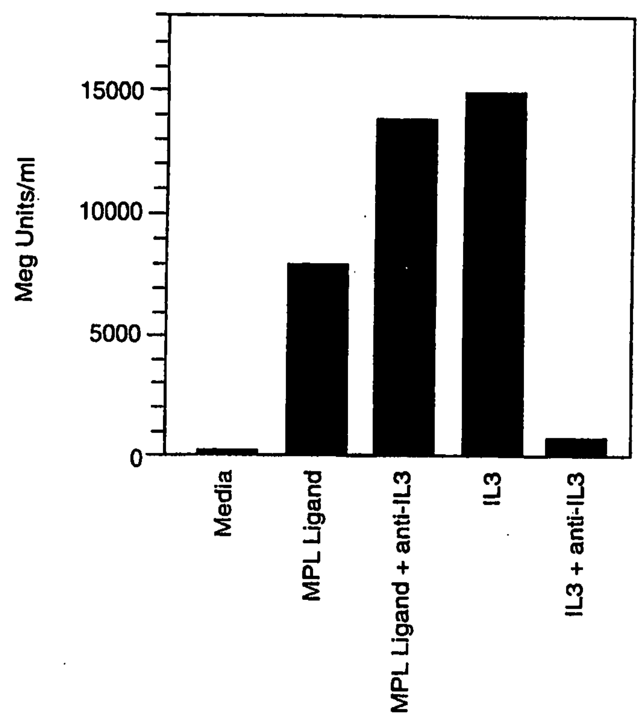

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FIG. 11A

```
  1  CAGGGAGCCACGCCAGCCAAGACACCCCGGCCAGAATGGAGCTGACTGAATTGCTCCTC   59
  1                                          MetGluLeuThrGluLeuLeuLeu   8

 60  GTGGTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGT  119
  9  ValValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCys   28

120  GACCTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATGTCCTTCACAGCAGACTGAGC  179
 29  AspLeuArgValLeuSerLysLeuLeuArgAspSerHisValLeuHisSerArgLeuSer   48

180  CAGTGCCCAGAGGTTCACCCTTTGCCTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGC  239
 49  GlnCysProGluValHisProLeuProThrProValLeuLeuProAlaValAspPheSer   68

240  TTGGGAGAATGGAAAACCCAGATGGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTG  299
 69  LeuGlyGluTrpLysThrGlnMetGluGluThrLysAlaGlnAspIleLeuGlyAlaVal   88

300  ACCCTTCTGCTGGAGGGAGTGATGGCAGCACGGGGACAACTGGGACCCACTTGCCTCTCA  359
 89  ThrLeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeuGlyProThrCysLeuSer  108

360  TCCCTCCTGGGGCAGCTTTCTGGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTC  419
109  SerLeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeu  128

420  CTTGGAACCCAGCTTCCTCCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATC  479
129  LeuGlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIle  148

480  TTCCTGAGCTTCCAACACCTGCTCCGAGGAAAGGTGCGTTTCCTGATGCTTGTAGGAGGG  539
149  PheLeuSerPheGlnHisLeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGly  168

540  TCCACCCTCTGCGTCAGGCGGGCCCCACCCACCACAGCTGTCCCCAGCAGAACCTCTCTA  599
169  SerThrLeuCysValArgArgAlaProProThrThrAlaValProSerArgThrSerLeu  188

600  GTCCTCACACTGAACGAGCTCCCAAACAGGACTTCTGGATTGTTGGAGACAAACTTCACT  659
189  ValLeuThrLeuAsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThr  208

660  GCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGGGATTCAGAGCCAAG  719
209  AlaSerAlaArgThrThrGlySerGlyLeuLeuLysTrpGlnGlnGlyPheArgAlaLys  228

720  ATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAAC  779
229  IleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsn  248

780  AGGATACACGAACTCTTGAATGGAACTCGTGGACTCTTTCCTGGACCCTCACGCAGGACC  839
249  ArgIleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr  268

840  CTAGGAGCCCCGGACATTTCCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCTC  899
269  LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsnLeu  288
```

FIG. 11B

```
900  CAGCCTGGATATTCTCCTTCCCCAACCCATCCTCCTACTGGACAGTATACGCTCTTCCCT  959
289  GlnProGlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeuPhePro  308

960  CTTCCACCCACCTTGCCCACCCCTGTGGTCCAGCTCCACCCCCTGCTTCCTGACCCTTCT  1019
309  LeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuProAspProSer  328

1020 GCTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAAT  1079
329  AlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThrHisSerGlnAsn  348

1080 CTGTCTCAGGAAGGGTAAGGTTCTCAGACACTGCCGACATCAGCATTGTCTCGTGTACAG  1139
349  LeuSerGlnGluGlyEnd                                            353
1140 CTCCCTTCCCTGCAGGGCGCCCCTGGGAGACAACTGGACAAGATTTCCTACTTTCTCCTG  1199
1200 AAACCCAAAGCCCTGGTAAAAGGGATACACAGGACTGAAAAGGGAATCATTTTTCACTGT  1259
1260 ACATTATAAACCTTCAGAAGCTATTTTTTTAAGCTATCAGCAATACTCATCAGAGCAGCT  1319
1320 AGCTCTTTGGTCTATTTTCTGCA  1342
```


FIG. 12A

```
1    AGGGAGCCACGCCAGCCAGACACCCCGGCCAGAATGGAGCTGACTGAATTGCTCCTCGTG  60
                                        MetGluLeuThrGluLeuLeuLeuVal  9

61   GTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGTGAC  120
10   ValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCysAsp  29

121  CTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATGTCCTTCACAGCAGACTGAGCCAG  180
30   LeuArgValLeuSerLysLeuLeuArgAspSerHisValLeuHisSerArgLeuSerGln  49

181  TGCCCAGAGGTTCACCCTTTGCCTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGCTTG  240
50   CysProGluValHisProLeuProThrProValLeuLeuProAlaValAspPheSerLeu  69

241  GGAGAATGGAAAACCCAGATGGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTGACC  300
70   GlyGluTrpLysThrGlnMetGluGluThrLysAlaGlnAspIleLeuGlyAlaValThr  89

301  CTTCTGCTGGAGGGAGTGATGGCAGCACGGGGACAACTGGGACCCACTTGCCTCTCATCC  360
90   LeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeuGlyProThrCysLeuSerSer  109

361  CTCCTGGGGCAGCTTTCTGGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTCCTT  420
110  LeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeu  129

421  GGAACCCAGCTTCCTCCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATCTTC  480
130  GlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIlePhe  149

481  CTGAGCTTCCAACACCTGCTCCGAGGAAAGGACTTCTGGATTGTTGGAGACAAACTTCAC  540
150  LeuSerPheGlnHisLeuLeuArgGlyLysAspPheTrpIleValGlyAspLysLeuHis  169

541  TGCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGGGATTCAGAGCCAA  600
170  CysLeuSerGlnAsnTyrTrpLeuTrpAlaSerGluValAlaAlaGlyIleGlnSerGln  189

601  GATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAA  660
190  AspSerTrpSerAlaGluProAsnLeuGlnValProGlyProAsnProArgIleProGlu  209

661  CAGGATACACGAACTCTTGAATGGAACTCGTGGACTCTTTCCTGGACCCTCACGCAGGAC  720
210  GlnAspThrArgThrLeuGluTrpAsnSerTrpThrLeuSerTrpThrLeuThrGlnAsp  229

721  CCTAGGAGCCCCGGACATTTCCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCT  780
230  ProArgSerProGlyHisPheLeuArgAsnIleArgHisArgLeuProAlaThrGlnPro  249

781  CCAGCCTGGATATTCTCCTTCCCCAACCCATCCTCCTACTGGACAGTATACGCTCTTCCC  840
250  ProAlaTrpIlePheSerPheProAsnProSerSerTyrTrpThrValTyrAlaLeuPro  269
```

FIG. 12B

```
 841  TCTTCCACCCACCTTGCCCACCCCTGTGGTCCAGCTCCACCCCCTGCTTCCTGACCCTTC   900
 270  SerSerThrHisLeuAlaHisProCysGlyProAlaProProProAlaSerEnd         289

 901  TGCTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAA   960
 961  TCTGTCTCAGGAAGGGTAAGGTTCTCAGACACTGCCGACATCAGCATTGTCTCGTGTACA  1020
1021  GCTCCCTTCCCTGCAGGGCGCCCCTGGGAGACAACTGGACAAGATTTCCTACTTTCTCCT  1080
1081  GAAACCCAAAGCCCTGGTAAAAGGGATACACAGGACTGAAAAGGGAATCATTTTTCACTG  1140
1141  TACATTATAAACCTTCAGAAGCTA  1164
```

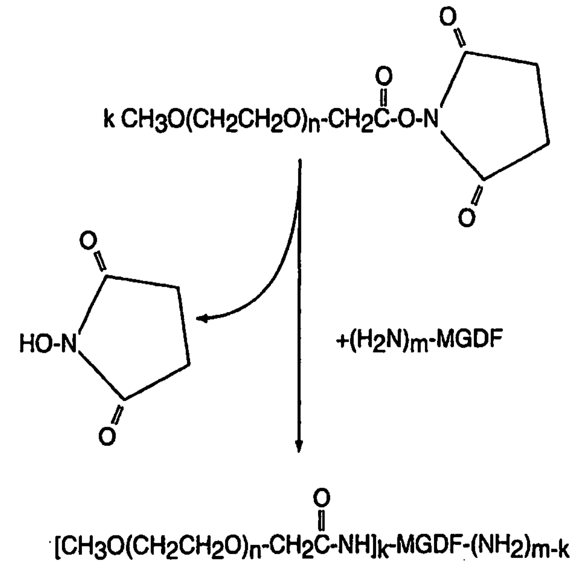

FIG. 13A

```
canine   1 MELTELLLVVMLLLTARLDPCLPAPPACDPRLLNKMLRDSHVLHSRLSQC 50
           ||||||||||||||||||. : ||||||| |:|.|:||||||||||||||
human    1 MELTELLLVVMLLLTARLTLSSPAPPACDLRVLSKLLRDSHVLHSRLSQC 50

        51 PDIYPLSTPVLLPAVDFSLGEWKTQKEQTKAQDVWGAVALLLDGVLAARG 100
           |::.||.||||||||||||||||||.|:|||||::|||.|||:||:||||
        51 PEVHPLPTPVLLPAVDFSLGEWKTQMEETKAQDILGAVTLLLEGVMAARG 100

       101 QLGPSCLSSLLGQLSGQVRLLLGALQGLLGTQLPPQGRTTTHKDPNAIFL 150
           ||||.|||||||||||||||||||||:||||||||||||||.|||||||||
       101 QLGPTCLSSLLGQLSGQVRLLLGALQSLLGTQLPPQGRTTAHKDPNAIFL 150

       151 SFQQLLRGKVRFLLLVAGPTLCAKQSQPTTAVPTNTSLFLTLRKLPNRTS 200
           |||:|||||||||:||:|.|||.:...||||||..|||.|||..||||||
       151 SFQHLLRGKVRFLMLVGGSTLCVRRAPPTTAVPSRTSLVLTLNELPNRTS 200

       201 GLLETNSSISARTTGSGLLKRLQGFRAKIPGLLNQTSRSLNQTPGHLSRT 250
           |||||| . |||||||||||: ||||||||||||||||||:|.||.|.|.
       201 GLLETNFTASARTTGSGLLKWQQGFRAKIPGLLNQTSRSLDQIPGYLNRI 250

       251 HGPLNGTHGLLPGLSLTALGAPDIPPGTSDMDALPPNLWPRYSPSPIHPP 300
           |: ||||:||:|| |  .||||||..|||| :.||||| | |||||.|||
       251 HELLNGTRGLFPGPSRRTLGAPDISSGTSDTGSLPPNLQPGYSPSPTHPP 300

       301 PGQYTLFSPLPTSPTPQNPLQPPPPDPSA.TANSTSPLLIAAHPHFQNLS 349
           .||||||.  || |||  .|:|  ||||| |:..||||| ....| ||||
       301 TGQYTLFPLPPTLPTPVVQLHPLLPDPSAPTPTPTSPLLNTSYTHSQNLS 350

       350 QEE 352
           ||:
       351 QEG 353
```

FIG. 13B

```
murine 1 MELTDLLLAAMLLAVARLTLSSPVAPACDPRLLNKLLRDSHLLHSRLSQC 50
human  1 MELTELLLVVMLLLTARLTLSSPAPPACDLRVLSKLLRDSHVLHSRLSQC 50

      51 PDVDPLSIPVLLPAVDFSLGEWKTQTEQSKAQDILGAVSLLLEGVMAARG 100
      51 PEVHPLPTPVLLPAVDFSLGEWKTQMEETKAQDILGAVTLLLEGVMAARG 100

     101 QLEPSCLSSLLGQLSGQVRLLLGALQGLLGTQLPLQGRTTAHKDPNALFL 150
     101 QLGPTCLSSLLGQLSGQVRLLLGALQSLLGTQLPPQGRTTAHKDPNAIFL 150

     151 SLQQLLRGKVRFLLLVEGPTLCVRRTLPTTAVPSSTSQLLTLNKFPNRTS 200
     151 SFQHLLRGKVRFLMLVGGSTLCVRRAPPTTAVPSRTSLVLTLNELPNRTS 200

     201 GLLETNFSVTARTAGPGLLSRLQGFRVKITPGQLNQTSRSPVQISGYLNR 250
     201 GLLETNFTASARTTGSGLLKWQQGFRAKI.PGLLNQTSRSLDQIPGYLNR 249

     251 THGPVNGTHGLFAGTSLQTLEASDISPGAFNKGSLAFNLQGGLPPSPSLA 300
     250 IHELLNGTRGLFPGPSRRTLGAPDISSGTSDTGSLPPNLQPGYSPSPTHP 299

     301 PDGH.TPPPPSPALPTTHGSPPQLHPLFPDPSTTMPNSTAPHPVTMYPHP 349
     300 PTGQYTLFPLPPTLPT...PVVQLHPLLPDPSAPTPTPTSPLLNTSYTHS 346

     350 RNLSQET 356
     347 QNLSQEG 353
```


FIG. 14



k, m and n are the same as defined in Figure 15.

FIG. 15

$$k\ CH_3O(CH_2CH_2O)_nCH_2CH_2C(O)H + (H_2N)_m\text{-MGDF}$$

$$\Big\downarrow NaCNBH_3$$

$$[CH_3O(CH_2CH_2O)_nCH_2CH_2CH_2\text{-HN}]_k\text{-MGDF-}(NH_2)_{m-k}$$

k - number of PEG molecules reacted with a molecule of MGDF.

n - degree of polymerization of PEG used in the reaction; e.g. n=2000 for PEG of MW=100 kD; n=40 for PEG of MW=2 kD.

m - total number of primary amino groups per MGDF molecule.

FIG. 16

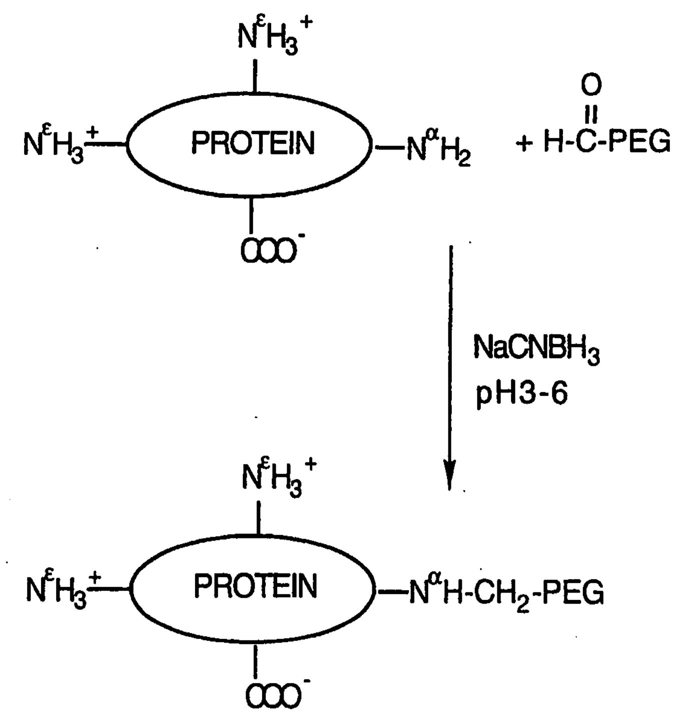


FIG. 17A

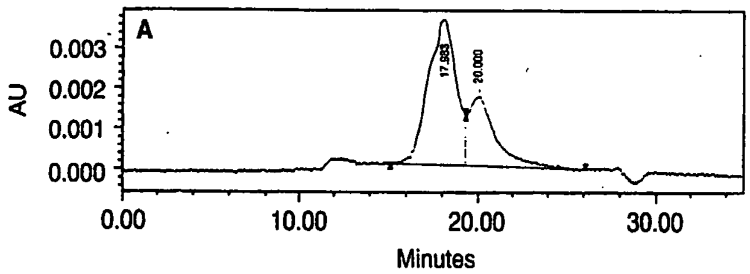


FIG. 17B

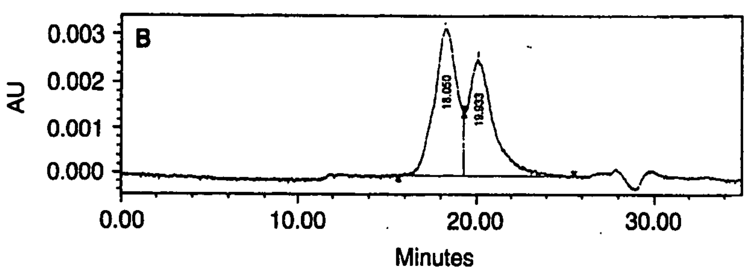


FIG. 17C

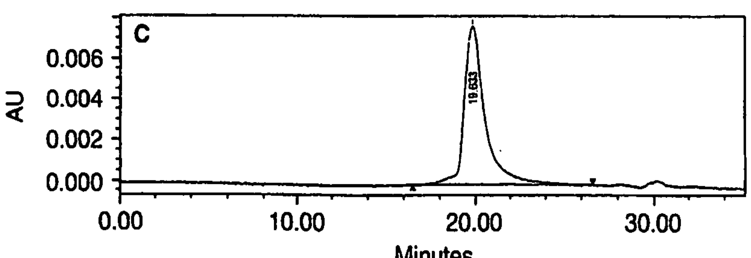


FIG. 18

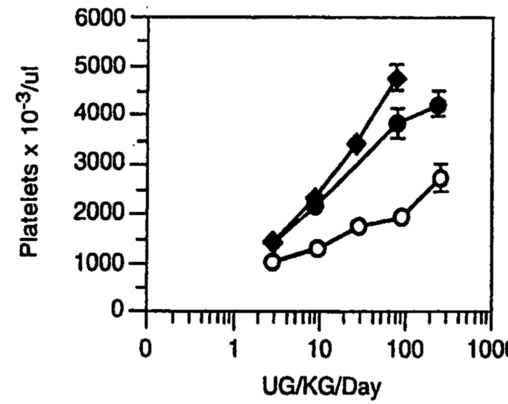


FIG. 19

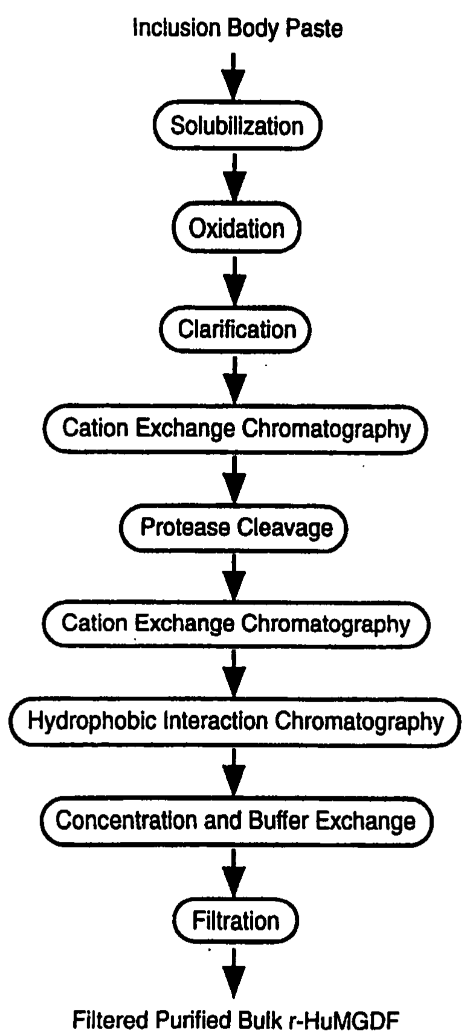

Inclusion Body Paste
Solubilization
Oxidation
Clarification
Cation Exchange Chromatography
Protease Cleavage
Cation Exchange Chromatography
Hydrophobic Interaction Chromatography
Concentration and Buffer Exchange
Filtration
Filtered Purified Bulk r-HuMGDF

FIG. 20

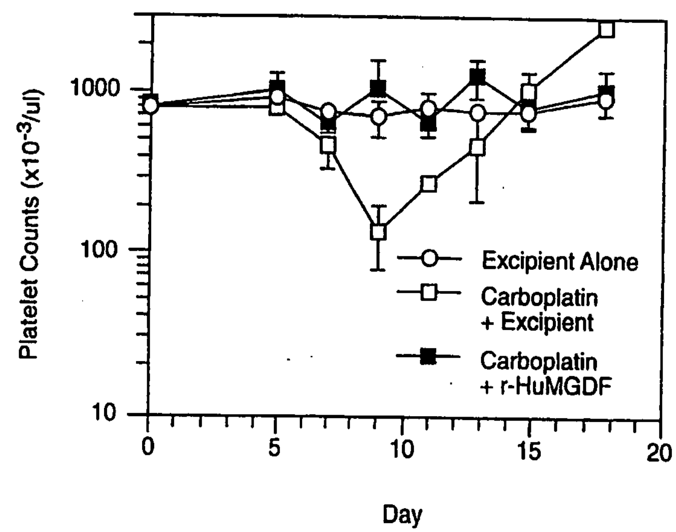

Platelet Counts (x10-3/ul)
1000
100
10
0
5
10
15
20
Day
Excipient Alone
Carboplatin + Excipient
Carboplatin + r-HuMGDF

FIG. 21

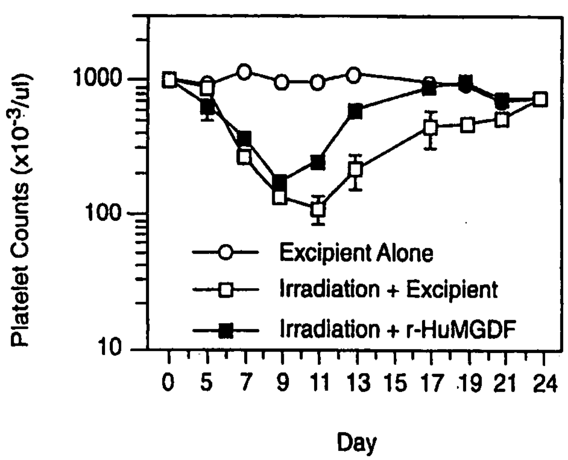

Platelet Counts (x10-3/ul)
1000
100
10
0 5 7 9 11 13 15 17 19 21 24
Day
Excipient Alone
Irradiation + Excipient
Irradiation + r-HuMGDF

FIG. 22

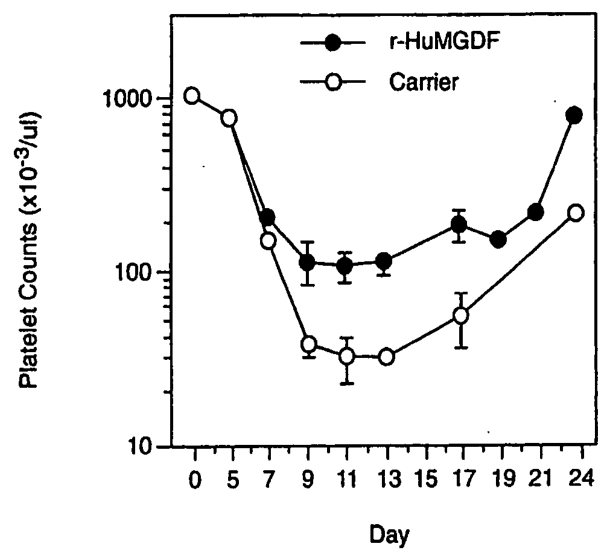

Platelet Counts (x10-3/ul)
1000
100
10
0 5 7 9 11 13 15 17 19 21 24
Day
r-HuMGDF
Carrier

FIG. 23

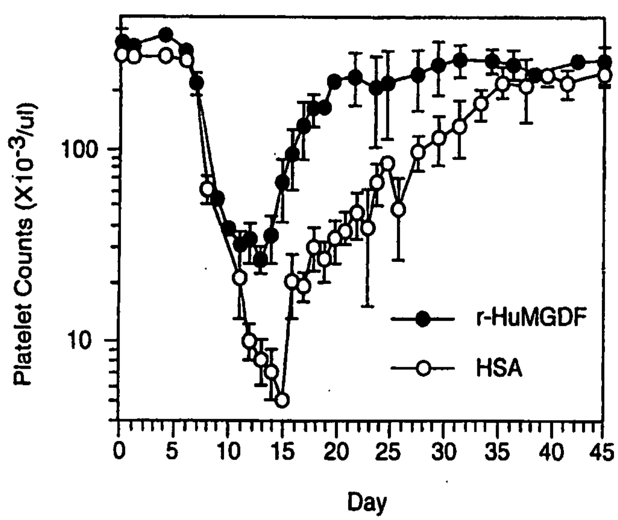
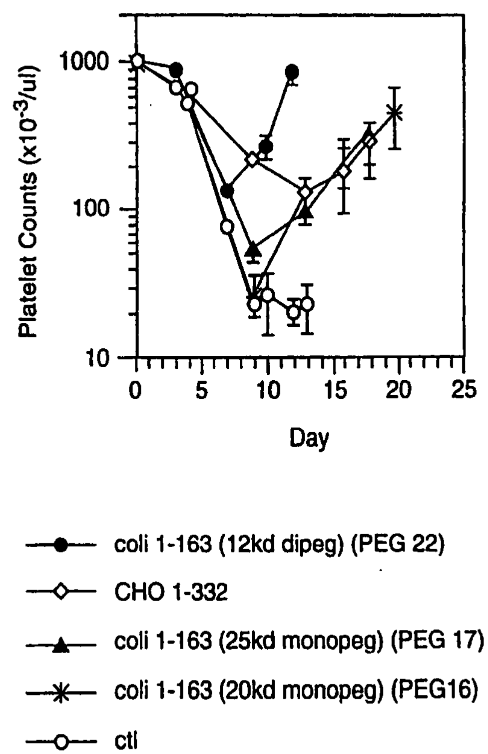

Platelet Counts (X10-3/ul)
100
10
0
5
10
15
20
25
30
35
40
45
Day
r-HuMGDF
HSA

FIG. 24


Platelet Counts (x10-3/ul)
1000
100
10
0
5
10
15
20
25
Day
coli 1-163 (12kd dipeg) (PEG 22)
CHO 1-332
coli 1-163 (25kd monopeg) (PEG 17)
coli 1-163 (20kd monopeg) (PEG16)
ctl

28/28

FIG. 25

ATG AAA AGT CCT GCA CCA CCT GCA TGT GAT TTA CGG GTC CTG
MET LYS SER PRO ALA PRO PRO ALA CYS ASP LEU ARG VAL LEU

TCT AAA CTG CTG CGC GAC TCT CAC GTG CTG CAC TCT CGT CTG
SER LYS LEU LEU ARG ASP SER HIS VAL LEU HIS SER ARG LEU

TCC CAG TGC CCG GAA GTT CAC CCG CTG CCG ACC CCG GTT CTG
SER GLN CYS PRO GLU VAL HIS PRO LEU PRO THR PRO VAL LEU

CTT CCG GCT GTC GAC TTC TCC CTG GGT GAA TGG AAA ACC CAG
LEU PRO ALA VAL ASP PHE SER LEU GLY GLU TRP LYS THR GLN

ATG GAA GAG ACC AAA GCT CAG GAC ATC CTG GGT GCA GTA ACT
MET GLU GLU THR LYS ALA GLN ASP ILE LEU GLY ALA VAL THR

CTG CTT CTG GAA GGC GTT ATG GCT GCA CGT GGC CAG CTT GGC
LEU LEU LEU GLU GLY VAL MET ALA ALA ARG GLY GLN LEU GLY

CCG ACC TGC CTG TCT TCC CTG CTT GGC CAG CTG TCT GGC CAG
PRO THR CYS LEU SER SER LEU LEU GLY GLN LEU SER GLY GLN

GTT CGT CTG CTG CTC GGC GCT CTG CAG TCT CTG CTT GGC ACC
VAL ARG LEU LEU LEU GLY ALA LEU GLN SER LEU LEU GLY THR

CAG CTG CCG CCA CAG GGC CGT ACC ACT GCT CAC AAG GAT CCG
GLN LEU PRO PRO GLN GLY ARG THR THR ALA HIS LYS ASP PRO

AAC GCT ATC TTC CTG TCT TTC CAG CAC CTG CTG CGT GGC AAA
ASN ALA ILE PHE LEU SER PHE GLN HIS LEU LEU ARG GLY LYS

GTT CGT TTC CTG ATG CTG GTT GGC GGT TCT ACC CTG TGC GTT
VAL ARG PHE LEU MET LEU VAL GLY GLY SER THR LEU CYS VAL

CGT CGG GCG CCG CCA ACC ACT GCT GTT CCG TCT TAA
ARG ARG ALA PRO PRO THR THR ALA VAL PRO SER STOP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03776

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL : 530/350, 387.3, 399; 536/23.1, 23.5; 435/320.1, 252.3, 243; 424/184.1; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/350, 387.3, 399; 536/23.1, 23.5; 435/320.1, 252.3, 243; 424/184.1; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, DIALOG - Biotech Files, GenEMBL sequence databases

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Volume 82, Number 5, issued 01 September 1993, N. Methia et al, "Oligodeoxynucleotides antisense to the proto-oncogene c-*mpl* specifically inhibit in vitro megakaryocytopoiesis", pages 1395-1401, see entire document.	1-83
Y	Blood, Volume 81, Number 11, issued 01 June 1993, M. Ogawa, "Differentiation and proliferation of hematopoietic stem cells", pages 2844-2853, see entire document.	1-83
Y	EMBO Journal, Volume 12, Number 7, issued 1993, R. C. Skoda et al, "Murine c-*mpl*: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal", pages 2645-2653, see entire document.	1-83

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search: 07 JULY 1995

Date of mailing of the international search report: 18 JUL 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
ELIZABETH C. KEMMERER
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03776

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 89, issued June 1992, I. Vigon et al, "Molecular cloning and characterization of *MPL*, the human homolog of the *v-mpl* oncogene: Identification of a member of the hematopoietic growth factor receptor superfamily", pages 5640-5644, see entire document.	1-83
Y	Cell, Volume 63, issued 21 December 1990, M. Souyri et al, "A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors", pages 1137-1147, see entire document.	1-83
Y	Blood, Volume 73, Number 5, issued April 1989, F. Wendling et al, "Factor-dependent erythropoietic progenitor cells in leukemia induced by the myeloproliferative leukemia virus", pages 1161-1167, see entire document.	1-83
Y	Leukemia, Volume 3, Number 7, issued July 1989, F. Wendling et al, "Myeloid progenitor cells transformed by the myeloproliferative leukemia virus proliferate and differentiate in vitro without the addition of growth factors", pages 475-480, see entire document.	1-83
Y	US, A, 5,087,448 (BURSTEIN) 11 February 1992, see entire document.	28-35, 65, 66, 76-83
Y	US, A, 5,032,396 (WILLIAMS) 16 July 1991, see entire document.	28-35, 65, 66, 76-83
Y	US, A, 5,264,209 (MIKAYAMA ET AL) 23 November 1993, see entire document.	36-83
Y	Critical Reviews in Drug Carrier Systems, Volume 9, Numbers 3 and 4, issued 1992, C. Delgado et al, "The uses and properties of PEG-linked proteins", pages 249-304, see entire document.	36-83

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03776

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/16, 38/18; C07H 21/04; C07K 1/12, 14/00, 14/435, 14/475; C12N 15/00, 15/09, 15/12

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-24 and 28-30, drawn to MDGF proteins, DNA, methods of expressing protein, and pharmaceutical compositions comprising the protein;

II. Claims 25-27, drawn to MDGF antibodies;

III. Claims 31-35, drawn to methods of administering the MDGF proteins;

IV. Claims 36-52, 62-70, and 76-78, drawn to MDGF derivatives and pharmaceutical compositions comprising same, irrespective of the method of making such;

V. Claims 53-61 and 71-75, drawn to a method of making MDGF derivatives;

VI. Claims 79-83, drawn to methods of administering MDGF derivatives.

The products of Groups I, II and IV do not share a special technical feature. For example, the MDGF proteins of Group I can be used other than to make the antibody of Group II, such as in therapy. The antibodies of Group II can be used other than to isolate the protein, such as in immunodiagnostics. The MDGF derivatives of Group IV can be used other than to make antibodies, such as in pharmaceutical compositions. Similarly, the methods of Groups III, V, and VI do not share a special technical feature, in that they require different compounds, and can be used to make different compounds. Although the protein of Group I can be used in the method of Group III, it can also be used in materially different processes, such as in antibody production. The derivatives of Group IV is related to the method of making same in Group V; however, the method is applicable to the modification of any one of a number of different proteins. The derivatives of Group IV are related to the method of using same of Group VI; however, the method can be practiced with MDGF proteins which have not been modified. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.